Vol. XLIV No. 8

August 1955

SCIENTIFIC EDITION

JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

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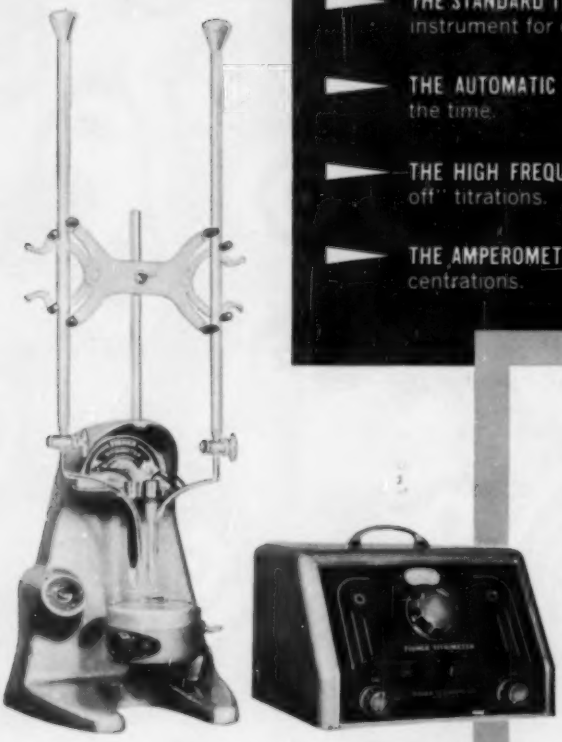

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Editorial and Executive Offices—2215 Constitution Avenue, N.W., Washington 7, D. C. Robert P. Fischelis, Secretary and General Manager.

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Entered as second class matter January 23, 1917, at the Post Office at Easton, Pennsylvania, under the Act of March 3, 1879, as 24 times a year; Scientific Edition monthly on the 5th: Practical Pharmacy Edition monthly on the 20th. Acceptance for mailing at a special rate of postage provided for in Section 1103, Act of October 3, 1917, authorized July 10, 1918.

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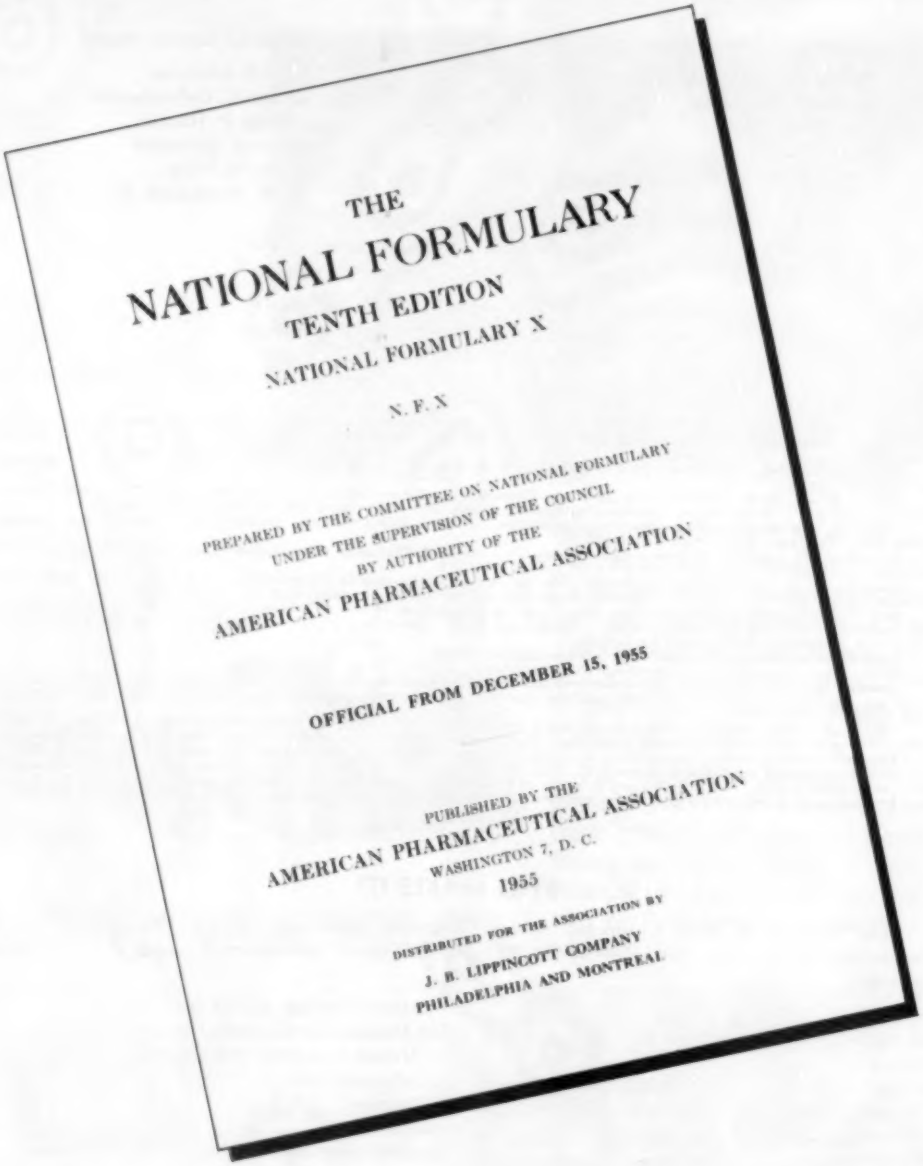

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VOLUME XLIV — *AUGUST, 1955* — NUMBER 8

CONSECUTIVE NO. 16

An Evaluation of Chemical Preservatives for Ophthalmic Solutions*

By C. A. LAWRENCE

Data are presented on the relative antimicrobial activities of seven commonly used chemical compounds that are used for preserving ophthalmic drug solutions from *Pseudomonas* and *Proteus* contaminations. In testing the compounds against 26 strains of *Pseudomonas aeruginosa* and four species of *Proteus* in a simple buffer solution, in the absence of an ophthalmic drug, the order of activity of the agents from high to low were found to follow the pattern: Benzalkonium chloride > phenylmercuric nitrate > phenol or chlorobutanol > parabens > thimerosal > phenylethyl alcohol. When the compounds were tested against some of the organisms in distilled water the order of activity from high to low was found to be as follows: Benzalkonium chloride > phenylmercuric nitrate > chlorobutanol > phenol or thimerosal > parabens > phenylethyl alcohol. When the preservatives were added to aqueous solutions of ophthalmic drugs experimentally contaminated with *Pseudomonas aeruginosa*, the order of activity of the compounds was: Benzalkonium chloride > chlorobutanol > phenol > parabens > phenylmercuric nitrate > phenylethyl alcohol or thimerosal. Several suggestions are given for the selection of a suitable preserving agent for ophthalmic drugs and certain procedures to be followed in the formulation of solutions under practical conditions in hospital and pharmacy routine.

A REVIEW of the recent literature (1) on the antibacterial effectiveness of various chemical preservatives for ophthalmic solutions will reveal considerable differences in opinions concerning the efficacy of any one particular compound that has been prescribed for the "self-sterilization" of ophthalmic drugs. This is notably the case in those instances in which *Pseudomonas* and *Proteus* organisms are involved as the contaminants in the compounding or subsequent use of the solutions.

An analysis of the data of a number of workers will show that some of the antibacterial agents in current use can be relied upon to provide "self-sterilizing" ophthalmic solutions when the examinations are made under carefully controlled laboratory conditions (1). Others have extended these studies under practical conditions of compounding prescriptions in the routine pharmacy practice (2, 3, 4). On the other hand, either through complete unawareness of these published data, or basing their opinions on isolated instances of ophthalmic infections due to improper use of chemical preservatives (inadequate concentrations, incompatibilities, etc.), the use of some of the latter has been contraindicated in multiple-dose units (5, 6, 7). To supplant the latter, recommendations have been made to have available single-dose containers of ophthalmic drugs without chemical preservatives, the solutions being sterilized by passage through a conventional bacterial filter (6). This is particularly the case in those instances where treatment by heat has an adverse effect upon the stability and pharmacologic activity of the ophthalmic drug.

* Received February 21, 1955, from the Los Angeles County Health Department and Department of Infectious Diseases, School of Medicine, University of California, Los Angeles.

Theodore and Feinstein have recently described the assembly and use of a sterile filtration unit for the preparation of ophthalmic solutions (5, 6). The installation and maintenance of a unit of this type is feasible in pharmaceutical manufacturing plants, in many of the larger pharmacy dispensing services in hospitals, and in certain independent prescription pharmacies catering to a large group of ophthalmologists. On the other hand, the majority of the individual pharmacies lack the funds, trained personnel, and equipment to maintain a sterilization unit, and it is in these instances where reliance is made on the use of a suitable chemical agent for preserving ophthalmic solutions.

Concerning the sources of contamination in ophthalmic solutions by *Pseudomonas* and *Proteus*, these may be attributed to (*a*) introduction of the latter by droppers contaminated from an infected eye of a patient (7); (*b*) a dropper that had been contaminated by being placed unprotected on a medicine cabinet shelf or on other surfaces (7); (*c*) the use of contaminated ophthalmic drugs in compounding; and (*d*) the use of contaminated distilled water used in the preparation of the solutions. The last named is probably the most likely source of contamination.

To avoid or correct the conditions cited above, infection from ophthalmic solutions, when due to contaminated droppers, can be prevented by using individual, disposable droppers (7). Conceivably, sterile ophthalmic drugs in powder form could be supplied by the manufacturer in which a "bactericidal" solvent is used in the final step of synthesis of the drug, with the drying process being carried out in a sterile atmosphere and the medicament than packaged in sterile containers. Since distilled water is usually prepared and collected in nonsterile containers and the water alone is known to support the growth of a variety of bacteria, notably of the *Pseudomonas* group, sterilization of the water soon after distillation, or at least before preparing ophthalmic solutions, should be the routine in sound pharmaceutical practice. This can be accomplished adequately by boiling, or, preferably, autoclaving the water soon after distillation.

Many of the publications favoring the use of, and/or objecting to the incorporation of, a particular type of chemical preserving agent in ophthalmic solutions have been expressions of opinions without any factual laboratory or clinical data to substantiate such opinions (5, 6, 7). Furthermore, in some instances where laboratory data are presented on the relative merits or shortcomings of a chemical compound in experimentally contaminated ophthalmic solutions, use is made of only a single strain of the organism under examination (2, 3, 4). Theodore and Feinstein (6) have indicated the existence of strains of *Pseudomonas* which vary in their degrees of susceptibility to the action of chlorobutanol. While they indicated that they were able to confirm an earlier observation by McCulloch (8), who found that 0.3% of the compound would prevent the growth of *Pseudomonas* in ophthalmic solutions, they found that "our investigations indicate that even weaker concentrations (0.125%) are effective against certain strains of *Pseudomonas* and *P. vulgaris*."

Although some of the more active chemical preservatives are known to be highly bacteriostatic in extremely low concentrations (quaternary ammonium surface-active germicides, mercurials), only a limited number of workers have taken under consideration the utilization of "antidotes," "neutralizers," or "inhibitors" to distinguish between the bactericidal and bacteriostatic activities of the "preserved" ophthalmic solutions under investigation.

A search of the literature on the laboratory evaluation of preservatives for ophthalmic solutions will reveal that Hughson and Styron (4) are probably the only investigators who gave consideration to the inactivation of the preservative (benzalkonium chloride) in the subculture medium by using sterile evaporated milk or rabbit blood.

Several chemical compounds have been used as antibacterial agents in ophthalmic solutions. Among these are chlorobutanol (6), phenylmercuric nitrate (9, 10) and benzalkonium chloride (1). Goldstein (11, 12) and others have added to this list phenol, phenylethyl alcohol (13, 14, 15), thimerosal, and a combination of methyl- and propylparaben. On the basis of the studies by Goldstein (11), and Goldstein and Ryan (12), all of the agents mentioned above (phenylmercuric nitrate was not included in the investigation) were found effective in preserving a large number of ophthalmic drugs which were compounded under normal pharmacy practice. The report also lists the various compatibilities and incompatibilities that can be expected with the several preservatives and the drugs.

The present study was undertaken to extend the work of Goldstein and others on this subject to include a large number of *Pseudomonas aeruginosa* strains and several species of *Proteus* organisms. An attempt was also made to distinguish between the bacteriostatic and bactericidal activities of several of the chemical compounds known to be highly bacteriostatic (benzalkonium chloride, thimerosal, phenylmercuric

nitrate) by the use of recognized inactivators in the testing procedure.

In a preliminary evaluation of the antibacterial activity of several chemical preserving agents, it appeared desirable to determine the time required for the recommended concentrations of the compounds to inhibit the development of *Pseudomonas* and *Proteus* cultures when the compounds were tested upon the latter in a standard buffer solution. Two of the most effective agents examined in the latter system were tested further in the presence of the same buffer solution along with several ophthalmic drugs. A final study was made wherein all of the preservatives were evaluated in the presence of several drugs dissolved in distilled water.

PROCEDURE I

The chemical "preservatives" examined and their respective concentrations used were as follows:

1. Benzalkonium chloride,	1:5,000 & 1:10,000
2. Chlorobutanol,	1:200
3. Methylparaben + Propylparaben,	1:625 1:5,000
4. Phenol,	1:200
5. Phenylethyl alcohol,	1:200
6. Phenylmercuric nitrate,	1:10,000 & 1:20,000
7. Thimerosal,	1:10,000 & 1:20,000

The buffer system[1] used as the diluent for the chemical compounds was prepared as follows: a stock concentrate was made with 34 Gm. of potassium dihydrogen phosphate (KH_2PO_4) dissolved in 500 ml. of distilled water and to this was then added 175 ml. of 4% NaOH. The solution was finally diluted to 1000 ml. with distilled water and adjusted to *p*H 7.2. One and one-quarter milliliters of the stock concentrate was diluted with 998.75 ml. of distilled water and sterilized at 15 lbs. steam pressure for thirty minutes. This diluting fluid was stored at room temperature until used.

Some of the *Pseudomonas* cultures and all of the *Proteus* species used in the study represented strains maintained in several laboratories in their culture collections and had been transplanted at periodic intervals on nutrient agar slants for several years. In addition to the former, a number of *Pseudomonas* strains used in the investigation were isolated recently from human sources. The origin of the cultures is indicated under Table I. All cultures were transferred daily for at least one week in beef extract broth (FDA medium, Circular 198) before being used in the study. In all instances the inoculum consisted of 0.5 ml. of a twenty-four-hour undiluted broth culture which had been incubated at 37°. The inoculum was added to 5 ml. of the test solution. A 4-mm. loopful of the treated organism suspension was transferred to approximately 10 ml. of sterile medium at the end of one-half, one, three, six, twenty-four, and forty-eight hours, and in some instances at the end of six days exposure to the chemical agents. The temperature at which the test was conducted was approximately 24–26° (room temperature). The subculture tubes were incubated at 37° for forty-eight hours and then kept at room temperature for an additional one hundred and twenty hours (total of seven days) before recording the presence or absence of visible growth.

Since chlorobutanol, the parabens, phenol, and phenylethyl alcohol do not exhibit any appreciable range of differences between their bactericidal and bacteriostatic concentration activities, transplants were made from these test solutions into plain FDA broth without any added inactivating substance. Thimerosal and phenylmercuric nitrate, on the other hand, are known to be highly bacteriostatic in relatively dilute solutions, and for this reason subcultures were made from these test solutions to tubes containing approximately 10 ml. of Brewer's fluid thioglycollate medium. Benzalkonium chloride also exhibits a bacteriostatic action in relatively high dilutions, thus the medium used to inactivate the latter property was beef extract broth to which was added 0.1% of Asolectin[2] and 0.7% Tween® 20.[3]

An examination of the results presented in Table I reveals the following:

(*a*) In most instances the preserving agents are effective in destroying the test organisms, provided they are permitted to act upon the organisms for a sufficient length of time. The latter factor is a variable one, being dependent upon the type of preservative used. With chlorobutanol, this may range from three to forty-eight hours with the various strains of *Pseudomonas*. Phenol shows a similar action from three to six hours and shows an action comparable to the paraben combination.

(*b*) Mention has been made in the literature of the possible use of phenylethyl alcohol as a preservative for ophthalmic solutions (13, 14). In light of the data presented in Table I the results appear disappointing with this agent. In most instances more than forty-eight hours were required for phenylethyl alcohol to render suspensions of *Pseudomonas* organisms sterile.

(*c*) Of the two mercurials examined, phenylmercuric nitrate was found to be considerably more active than thimerosal in its effects against the various strains of *Pseudomonas*. It is of interest to note, moreover, that the factor of dilutions of these two compounds does not appear to influence the activity of the compounds to any appreciable degree in the concentrations tested. Thus, fairly comparable results were obtained with the 1:10,000 and 1:20,000 dilutions of these agents.

(*d*) The results obtained with the quaternary ammonium surface-active germicide, benzalkonium chloride, confirm in part the published findings of others on the high germicidal activity of this compound upon organisms involved in contaminated ophthalmic solutions (2-4). Under the conditions of this study this agent was found to be the most effective preserving agent tested against the majority of the 26 strains of *Pseudomonas aeruginosa*.

(*e*) In general, all of the preservatives examined in this investigation were found to be more active against the several species of *Proteus* than against the *Pseudomonas* cultures.

[1] Formula used in the preparation of "Dilution Water" for the microbiological examination of milk and cream, "Standard Methods for the Examination of Dairy Products" 10th ed., American Public Health Association, 1953, page 95.

[2] Asolectin, Associated Concentrates, Inc., Atlanta, Ga.

[3] Tween-20, Atlas Powder Company, Wilmington, Del.

TABLE I.—EFFECTS OF SEVERAL OPHTHALMIC PRESERVATIVES ON PSEUDOMONAS AND PROTEUS

Organism Strain	Preservatives—First Appearance of "No Growth" in Subculture Tube: h = hour; d = days									
	1a[a]	1b	2	3	4	5	6a	6b	7a	7b
1. Ps-RS[b]	−1/2h[c]	−1/2h	6h	6h	6h	6d	1h	1h	3h	24h
2. Ps-R1	−1/2h	−1/2h	6h	6h	3h	6d	1h	1h	3h	24h
3. Ps-R2	−1/2h	3h	48h	6h	6h	6d	1h	1h	3h	24h
4. Ps-R3	−1/2h	−1/2h	3h	3h	3h	6d	1h	1h	3h	6h
5. Ps-R4	−1/2h	−1/2h	48h	6h	6h	6d	1h	1h	3h	24h
6. Ps-L	−1/2h	−1/2h	6h	6h	6h	6d	1h	1h	3h	24h
7. Ps-U21-a	−1/2h	3h	6h	6h	6h	6d	3h	3h	24h	24h
8. Ps-U21-2	−1/2h	−1/2h	3h	3h	3h	48h	1h	3h	6h	6h
9. Ps-U21-3	−1/2h	1h	3h	3h	3h	48h	1h	1h	3h	3h
10. Ps-U21-6	−1/2h	−1/2h	3h	3h	6h	6d	1h	1h	6h	24h
11. Ps-U21-7	−1/2h	6h	6h	6h	6h	6d	3h	3h	6h	24h
12. Ps-U21-11	−1/2h	−1/2h	3h	6h	6h	6d	3h	3h	6h	6h
13. Ps-51R	−1/2h	−1/2h	3h	6h	6h	6d	3h	3h	24h	24h
14. Ps-51S	−1/2h	−1/2h	24h	6h	6h	6d	1h	3h	24h	24h
15. Ps-52R	−1/2h	−1/2h	3h	3h	3h	6d	1h	3h	6h	24h
16. Ps-52S	−1/2h	−1/2h	6h	6h	3h	6d	3h	6h	6h	48h
17. Ps-53	−1/2h	−1/2h	3h	6h	6h	6d	3h	3h	6h	3h
18. Ps-Th-30	−1/2h	3h	1h	3h	1h	6d	1h	3h	24h	6h
19. Ps-U5	−1/2h	−1/2h	6h	6h	3h	48h	1h	1h	6h	6h
20. Ps-U	−1/2h	1h	3h	3h	3h	48h	−1/2h	3h	24h	24h
21. Ps-W	−1/2h	−1/2h	1h	3h	1h	6d	3h	1h	24h	24h
22. Ps-J	−1/2h	−1/2h	6h	6h	6h	6d	6h	6h	24h	24h
23. Ps-R	−1/2h	−1/2h	3h	6h	6h	6d	6h	6h	24h	24h
24. Ps-C	−1/2h	3h	3h	3h	6h	6d	3h	6h	24h	24h
25. Ps-D	−1/2h	3h	−1/2h	3h	3h	6d	1h	1h	3h	3h
26. Ps-G	−1/2h	−1/2h	3h	6h	48h	6d	3h	3h	6h	24h
1. P.[d] vulg. R1	−1/2h	−1/2h	−1/2h	1h	1h	6h	−1/2h	1h	6h	6h
2. P. vulg. UM	−1/2h	−1/2h	3h	3h	3h	48h	3h	3h	24h	24h
3. P. vulg. X2	−1/2h	−1/2h	−1/2h	3h	1h	6h	−1/2h	1h	6h	6h
4. P. mirab.	−1/2h	−1/2h	1h	3h	1h	24h	3h	3h	24h	24h
5. P. morgani	−1/2h	−1/2h	3h	6h	3h	24h	1h	1h	24h	24h

[a] 1a = 1:5,000 benzalkonium chloride; 1b = 1:10,000 benzalkonium chloride; 2 = 0.5% chlorobutanol; 3 = 0.16% methyl-+0.02% propyl-paraben; 4 = 0.5% phenol; 5 = 0.5% phenylethyl alcohol; 6a = 1:10,000 phenylmercuric nitrate; 6b = 1:20,-000 phenylmercuric nitrate; 7a = 1:10,000 thimerosal; 7b = 1:20,000 thimerosal.

[b] Ps = *Pseudomonas aeruginosa* strains; strains 1–5 from the Winthrop-Stearns, Inc., Rensselaer, N. Y. culture collection; No. 6 isolated from a patient in the Los Angeles Co. Health Dept.; Nos. 8–12 from the culture collection of Dr. E. Schultz, Dept. Bacteriology and Experimental Pathology, School of Medicine, Stanford University; Nos. 13–17 from Dr. W. L. Gaby, Department of Bacteriology, Hahnemann Medical School, Philadelphia; Nos. 18–26 from Dr. J. W. Dedrick, Communicable Disease Division, Los Angeles Co. General Hospital (all strains in this group are recent isolates from humans).

[c] −1/2h represents no growth in the first subculture tube tested at the shortest time period of one-half (1/2h) hour of exposure of the organisms to the preservative.

[d] P = Proteus species; Nos. 1, 3, 4, and 5 from the Winthrop-Stearns, Inc., culture collection; No. 2 from the culture collection of the Department of Bacteriology, University of Michigan.

PROCEDURE II

With the evidence presented in Table I of the superiority of phenylmercuric nitrate and benzalkonium chloride over the other chemical agents, in their ability to destroy *Pseudomonas* and *Proteus* organisms, it appeared desirable to examine these two compounds against several strains of *Pseudomonas* in the presence of several ophthalmic drugs. The medicaments tested and their respective concentrations used in the experiment are as follows:

1. Atropine sulfate.....1.0%
2. Homatropine hydrobromide.....1.0%
3. Neosynephrine hydrochloride....1.0%
4. Penicillin "G" potassium.....20,000 u./ml.
5. Pilocarpine hydrochloride.....1.0%
6. Scopolamine hydrobromide.....1.0%
7. Tetracaine hydrochloride.....1.0%

Sterile buffer solution (Procedure I) was used as the diluting fluid for the drugs and a sufficient amount of benzalkonium chloride was added to the latter to provide a 1:5,000 and a 1:10,000 concentration of the germicide. In the phenylmercuric nitrate series the final concentrations of this preservative were 1:10,000 and 1:20,000. The two ranges of concentrations of the two compounds were included in the test for they appear to cover the ranges that are usually used in the compounding of ophthalmic solutions. A control series of tubes, which consisted of the ophthalmic drugs alone without a preservative, was also used in the present study.

Four strains of *Pseudomonas aeruginosa* were selected for this experiment. These included the Ps-RS, Ps-R3, Ps-R4, and Ps-W organisms. The age of the cultures, amount of inoculum, subculture media, and incubation of the transplants were the same as described in the previous experiment. A single digression from the latter is represented in the third period of transfer which in the present study was carried out at the end of four instead of three hours. The results of this investigation are given in Table II and may be summarized as follows:

(*a*) From the data given in Table II it is evident that in the usual recommended concentration of 1:5,000 of benzalkonium chloride, this compound is more effective in destroying *Pseudomonas aeruginosa* organisms in the presence of the several ophthalmic drugs than is the commonly used concentration of phenylmercuric nitrate (1:20,000). The data indicate, moreover, that in using comparable concentrations of both preservatives (1:

TABLE II.—EFFECTS OF BENZALKONIUM CHLORIDE AND PHENYLMERCURIC NITRATE ON PSEUDOMONAS AERUGINOSA IN EXPERIMENTALLY CONTAMINATED OPHTHALMIC SOLUTIONS

Drug	Organism Strain	Drug Control	Preservatives with Ophthalmic Drug—First Appearance of "No Growth" in Subculture Tube: h = hour; d = day			
			Benzalkonium Chloride		Phenylmercuric Nitrate	
			1:5,000	1:10,000	1:10,000	1:20,000
Atropine	Ps-RS	6d+[a]	−1/2h	−1/2h	−1/2h	−1/2h
SO_4	Ps-R3	6d+	−1/2h	−1/2h	−1/2h	−1/2h
1%	Ps-R4	6d+	−1/2h	−1/2h	−1/2h	−1/2h
	Ps-W	6d+	−1/2h	−1/2h	−1/2h	−1/2h
Homatropine	Ps-RS	6d+	−1/2h	1h	24h[b]	4h[b]
HBr	Ps-R3	6d+	−1/2h	−1/2h	4h	4h
1%	Ps-R4	6d+	−1/2h	−1/2h	4h	4h
	Ps-W	6d+	−1/2h	1h	4h	4h
Neosynephrine	Ps-RS	6d+	−1/2h	−1/2h	1h	−1/2h
HCl	Ps-R3	6d+	−1/2h	−1/2h	−1/2h	1h
1%	Ps-R4	6d+	−1/2h	−1/2h	1h	−1/2h
	Ps-W	6d+	−1/2h	−1/2h	1h	1h
Penicilllin G	Ps-RS	6d+	−1/2h	1h	24h	24h
Potassium	Ps-R3	6d	−1/2h	1h	4h	24h
20,000 u./ml.	Ps-R4	6d	−1/2h	−1/2h	4h	24h
	Ps-W	6d	−1/2h	4h	4h	24h
Pilocarpine	Ps-RS	6d+	−1/2h	−1/2h	4h	4h
HCl	Ps-R3	6+	−1/2h	−1/2h	1h	1h
1%	Ps-R4	6d+	−1/2h	4h	4h	4h
	Ps-W	6d+	−1/2h	−1/2h	1h	4h
Scopolamine	Ps-RS	6d+	−1/2h	−1/2h	24h	24h
HBr	Ps-R3	6d+	−1/2h	−1/2h	4h	4h
1%	Ps-R4	6d+	−1/2h	−1/2h	24h	24h
	Ps-W	6d+	−1/2h	−1/2h	24h	24h
Tetracaine	Ps-RS	−1/2h	−1/2h	−1/2h	−1/2h[c]	−1/2h
HCl	Ps-R3	−1/2h	−1/2h	−1/2h	−1/2h	−1/2h
1%	Ps-R4	−1/2h	−1/2h	−1/2h	−1/2h	−1/2h
	Ps-W	−1/2h	−1/2h	−1/2h	−1/2h	−1/2h

[a] The figure "+" following 6d indicates that a subculture made on the 6th (and final day of the experiment) showed the presence of viable organisms. The figure "−" preceding "1/2h" indicates that no growth appeared in the first subculture made at the earliest transfer period of one-half hour.

[b] Denotes an opalescence, or incompatibility, between phenylmercuric nitrate in both the 1:10,000 and 1:20,000 dilutions of the latter and homatropine.

[c] An opalescence with phenylmercuric nitrate, 1:10,000 but not 1:20,000 and tetracaine.

10,000), the quaternary ammonium compound is more active than the mercurial under the conditions of this experiment.

(*b*) The results obtained with tetracaine hydrochloride are noteworthy in that this drug alone, in the absence of an added chemical preservative, possesses a definite antimicrobial activity against *Pseudomonas aeruginosa* and possibly other microorganisms.[4]

PROCEDURE III

While the previous study was in progress, attention was directed to the fact that some clinicians are prescribing ophthalmic formulations that are prepared simply with distilled water rather than in a buffer system. Furthermore, since the various preservatives examined in Procedure II were all tested in a buffer solution of the drugs, conceivably the buffer itself may have had some adverse effect on the germicidal activity of one or more of the agents. To investigate these possibilities an experiment was designed in which distilled water was used in place of a buffer system and the study repeated by duplicating the conditions of Procedure II. Use of all the preservatives listed in Procedure I was made in obtaining the data presented in Table III. A single inactivating medium was used for subculturing from the test solutions of benzalkonium chloride and the two mercurials. This medium is essentially that described by Armbruster and Ridenour (16), with the exception that no additional agar was added to the formula (excepting that which is present in Brewers' fluid thioglycollate medium). The composition of the medium is as follows:

Thioglycollate fluid medium	29.5 Gm.
Tween® 20	30.0 ml.
Asolectin	2.0 Gm.
Distilled water	1,000 ml.
*p*H 7.0	

With the exceptions of benzalkonium chloride, thimerosal, and phenylmercuric nitrate, the concentrations of the preservatives used in this study were the same as that used in Procedure I. The former three agents were used in single concentrations of 1:5,000, 1:10,000, and 1:10,000, respectively. Also, with the exception of penicillin, the concentrations of ophthalmic drugs used in this test were the same as that used in Procedure II, 1%. The amount of the antibiotic tested in the present series was 40,000 u./ml. of distilled water alone, or the same amount in the latter with a preserving agent. The results of this study are presented in Table III.

[4] Since the pure drug, in crystalline form, was used in this study, the antibacterial activity can be attributed directly to the drug itself and not entirely to the vehicles or other agents used in tetracaine formulations that have been studied by Scigliano and Skolaut (17), and McPherson and Wood (3).

TABLE III.—EFFECTS OF SEVERAL PRESERVATIVES ON PSEUDOMONAS ABRUGINOSA IN EXPERIMENTALLY CONTAMINATED OPHTHALMIC SOLUTIONS

Drug	Organism Strain	Drug Control	Preservatives with Ophthalmic Drug—First Appearance of "No Growth" in Subculture Tube: h = hour; d = day													
			1[a]	1a	2	2a	3	3a	4	4a	5	5a	6	6a	7	7a
Atropine	Ps-RS	6d+	−½h	−½h	4h	−½h	48h	−½h	24h	−½h	6d+	1h	4h	1h	24h	6h
SO_4	Ps-R3	6d+	−½h	−½h	6h	−½h	24h	1h	24h	1h	6d+	1h	1h	1h	6h	4h
1%	Ps-R4	6d+	−½h	−½h	4h	−½h	48h	1h	6h	−½h	48h	1h	1h	1h	48h	24h
	Ps-W	6d+	−½h	−½h	6h	−½h	24h	−½h	24h	1h	6d+	4h	4h	−½h	6h	4h
Homatropine	Ps-RS	6d+		−½h		4h		4h		1h		24h		6h		4h
HBr	Ps-R3	6d+		−½h		1h		4h		−½h		4h		4h		4h
1%	Ps-R4	6d+		−½h		−½h		4h		1h		4h		24h		6h
	Ps-W	6d+		−½h		1h		4h		−½h		4h		4h		4h
Neosynephrine	Ps-RS	6d+		−½h		1h		1h		4h		6h		4h		24h
HCl	Ps-R3	6d+		−½h		4h		4h		4h		6h		1h		4h
1%	Ps-R4	6d+		−½h		4h		4h		4h		4h		4h		24h
	Ps-W	6d+		−½h		4h		4h		4h		4h		4h		6h
Penicillin G Potassium	Ps-RS	6d+		−½h		4h		4h		6h		6d		6h		48h
40,000 u./ml.	Ps-R3	6d+		−½h		4h		4h		4h		6d		4h		6h
	Ps-R4	6d+		−½h		4h		4h		4h		48h		4h		6d
	Ps-W	6d+		−½h		6h		4h		4h		6d		6h		24h
Pilocarpine	Ps-RS	6d+		−½h		1h		4h		4h		6d		24h		24h
HCl	Ps-R3	6d+		−½h		1h		4h		1h		6h		4h		24h
1%	Ps-R4	6d+		−½h		1h		6h		1h		6d		24h		24h
	Ps-W	6d+		−½h		1h		6h		1h		24h		24h		24h
Scopolamine	Ps-RS	6d+		−½h		1h		4h		4h		24h		24h[b]		24h
HBr	Ps-R3	6d+		−½h		1h		4h		4h		24h		6h		24h
1%	Ps-R4	6d+		−½h		1h		4h		4h		24h		48h		48h
	Ps-W	6d+		−½h		4h		1h		1h		24h		6h		24h
Tetracaine	Ps-RS	−½h		−½h		−½h		−½h		−½h		−½h		−½h		−½h
HCl	Ps-R3	−½h		−½h		−½h		−½h		−½h		−½h		−½h		−½h
1%	Ps-R4	−½h		−½h		−½h		−½h		−½h		−½h		−½h		−½h
	Ps-W	−½h		−½h		−½h		−½h		−½h		−½h		−½h		−½h

[a] Legend—Figure 1 represents control of benzalkonium chloride 1:5,000 in water while 1a the same concentration of the latter with the ophthalmic drugs; 2 the control on 0.5% chlorobutanol and 2b the latter with the drugs; 3 the control on 0.16% methyl- plus 0.02% propylparaben and 3a the latter with drugs; 4 the control on 0.5% phenol and 4a the latter with drugs; 5 the control on 0.5% phenylethylalcohol and 5a the latter with drugs; 6 the control on phenylmercuric nitrate and 6a the latter with drugs; 7 on thimerosal, 1:10,000, and 7a the latter with drugs. In the table of results the figure "+" following 6d indicates that a subculture made on the sixth day (and final day of the experiment) showed presence of viable organisms. The figure "−" preceding "½h" indicates that no growth appeared in the first subculture made at the earliest transfer period of one-half hour.

[b] An opalescent solution, indicative of a chemical incompatibility, resulted when 1:10,000 phenylmercuric nitrate was added to the 1% solution of scopolamine.

In summarizing the data given in the table it may be noted that in the order of activity from high to low the preservatives may be listed in the following manner:

Control (Preservatives Alone)	Preservatives with Ophthalmic Drugs
1. Benzalkonium chloride	1. Benzalkonium chloride
2. Phenylmercuric nitrate	2. Chlorobutanol
3. Chlorobutanol	3. Phenol
4. Phenol and/or thimerosal	4. Parabens
5. Parabens	5. Phenylmercuric nitrate
6. Phenylethyl alcohol	6. Phenylethyl alcohol and/or thimerosal

Another point worth noting from the data is that there appears to be a synergistic action between several of the preservatives and the ophthalmic drugs, for a greater degree of antibacterial activity may be noted when the combinations are used than that observed with the preservatives alone. The exceptions to this phenomenon are in the tests with the two mercurials, thimerosal and phenylmercuric nitrate. In these instances, in addition to the apparent incompatibility between phenylmercuric nitrate and scopolamine (opalescence), some of the antibacterial activity noted with the mercurials in distilled water alone, is diminished upon combining them with the various ophthalmic drugs.

An examination of the results obtained with phenylethyl alcohol will reveal that when tested alone in distilled water most of the strains of *Pseudomonas aeruginosa* survived exposure to this compound through the six-day period of treatment, or the extreme time limit of the study. On the other hand, when examined in the presence of several of the ophthalmic drugs there was some evidence of a preserving action of the compound under the conditions of this experiment.

The data obtained with benzalkonium chloride in the present and the preceding studies of this investigation, confirm and extend the observations of others on the value of this agent as a preservative for ophthalmic solutions in which *Pseudomonas aeruginosa* is often found as the contaminating organism (2–4, 9, 10).

DISCUSSION

Present official regulations (18) require sterility for all ophthalmic solutions prepared by pharmaceutical manufacturers. Under the present ruling, however, hospitals and retail pharmacies are exempted from the regulations because of the problems involved in preparing sterile ophthalmic solutions, especially by filtration procedures. Thus, until universal enforcement of the preparation of solutions by the latter methods can be adopted in accordance with such regulations, the use of chemical agents for preserving ophthalmic solutions appears to be the only alternative in the prevention of serious ophthalmic infections.

In the selection of a suitable chemical agent as a preservative, consideration should be given to several requirements that should be inherent and sought for in its adoption. Some of the important features are the following:

1. Chemically compatible with the ophthalmic drug.
2. Have no effect on the pharmacologic activity of the ophthalmic drug.
3. Should have a wide bacteriostatic (fungistatic, etc.) and preferably a bactericidal (fungicidal, etc.) activity against organisms encountered in the routine compounding of ophthalmic solutions.
4. Should be readily soluble in distilled water and certain buffer solutions.
5. Should not significantly alter the *p*H and tonicity of the ophthalmic solution.
6. Should be nontoxic and nonirritating to ocular tissues in the concentrations used for preserving ophthalmic drugs.
7. Should remain active and stable on storage under normal conditions.

Of all the chemical preservatives that have been used in the past, as well as those in current use today, there is no single agent that will satisfactorily meet all the criteria listed above. In the selection of the preservative for ophthalmic solutions, therefore, one must be guided by his own particular experiences with the compound, or by the published experiences of others. Two of the latter reports on compatibilities and incompatibilities of ophthalmic drugs with preservatives, are that of Lawrence (1) on benzalkonium chloride, and Hind and Szekely (9) on phenylmercuric nitrate. A general consideration of the same subject with other chemical compounds and ophthalmic drugs has been presented in the excellent publications of Goldstein (11) and Goldstein and Ryan (12). The present report and the many studies of others (2–4, 9, 17) describe the antibacterial activities that can be expected from the use of the various ophthalmic preservatives. This information and the other specifications set forth in the previous paragraph can be used as a guide in selecting a compound to be used in the formulation of "self-sterilizing" ophthalmic solutions.

Only a limited number of studies have been made in which chemical preservatives have been evaluated under conditions which can be encountered in the compounding of ophthalmic solutions in hospitals and pharmacies. Krause, Dauer, and Guth (2) examined several solutions from a retail pharmacy in which no preservative was used in prescriptions of pilocarpine and atropine and found the solutions heavily contaminated. On the other hand, in testing several solutions prepared in a hospital pharmacy in which 1:5,000 benzalkonium chloride was added as the preservative and using no other precautions to avoid bacterial contamination, all of the solutions were found to be sterile.

McPherson and Wood (3), and Hughson and Styron (4) prepared a number of ophthalmic solutions in which 1:5,000 benzalkonium chloride was added as the preserving agent. In the study conducted by the former investigators, the ophthalmic solutions were prepared in a buffer system without any other treatment to effect sterilization, prior to being placed in use in a hospital ward. The 27 solutions were cultured two and four weeks after being put in use and on both occasions all of the solutions were found to be sterile. In the same study, ophthalmic solutions were prepared in the same buffer system without the preservative, but in this instance the solutions were sterilized by autoclaving prior to use. Once in use these solutions were dis-

carded after a period of one week. Cultures made at this time showed an appreciable percentage of them to be contaminated. In addition to benzalkonium chloride, Goldstein has found that the other preservatives used in the present investigation are effective in preventing the development of mold growths in ophthalmic solutions under normal conditions of compounding the solutions.

The occasional reports in the literature indicating that ophthalmic solutions have been found contaminated with *Pseudomonas* and *Proteus* organisms, even though a preservative is present in the solution (19, 20), is sufficient cause for some consideration of a routine practice to be followed in hospitals and pharmacies in the formulation of such solutions with a minimal number or complete absence of viable microorganisms. Some of the more important factors that should be taken under consideration to achieve this are the following:

1. When available, use sterile ophthalmic drug powders.
2. Weighings of powders should be done in an aseptic manner on sterile, or, at least, on clean equipment and in a clean atmosphere.
3. Sterile distilled water (boiled, or preferably autoclaved) should be used consistently in the compounding of ophthalmic solutions. This also applies to buffer solutions when used as the diluent in the prescription.
4. Use of sterile dispensing bottles or containers. An alternative to this is rinsing the container with boiled or distilled water followed by treatment with a suitable chemical sterilizing agent.
5. Use of disposable droppers. Where the factor of cost prohibits this practice, use a "screw-on" bottle dropper, which is returned to the ophthalmic solution when not in use, instead of being placed on a contaminated shelf or other surface. Preliminary treatment of the dropper before dispensing the ophthalmic solution from the pharmacy can be carried out as in No. 4 above.
6. Use of a suitable chemical preservative which most nearlyful fills the requirements set forth in this report.

On the basis of the results presented in this investigation and the published findings of others (11, 12), all of the chemical agents used in this study can be expected to provide "self-sterilizing" ophthalmic solutions when due consideration is given to such important factors as: (*a*) time of exposure of the contaminating microorganisms to the antibacterial activity of the preparation; (*b*) numbers, types, species, and strains of microorganisms encountered in the ingredients of the formulation and the glassware used in compounding the prescription; and (*c*) the chemical and pharmacologic compatibilities and incompatibilities between the chemical preservative and the ophthalmic drugs.

A concluding note to this discussion should call attention to the results obtained on benzalkonium chloride, which confirm the data obtained by others on the same agent, in which one can demonstrate a high degree of bactericidal activity when the compound is used as a preserving agent in grossly contaminated ophthalmic solutions containing *Pseudomonas* and *Proteus* organisms. These findings are at variance with the published opinions of Theodore and Feinstein (5, 6) in which the statements are made, "The cationic wetting agents (benzalkonium, cetyl benzalkonium, and Phemerol®) appear to be of questionable value for use as preservatives. Their bacteriostatic effect is too variable and, at times unreliable, especially against *Ps. aeruginosa*." Additional reasons for avoiding the use of quaternary ammonium surface-active germicides are given in the publications of the latter authors. King (7) refers to the comments of Theodore and adds to the above remarks, "They (quaternaries) are not advised by many authors for use against *Pseudomonas* and are not recommended in the New and Nonofficial Remedies published by the Council of Pharmacy of the American Medical Association."[1]

In referring to the official publication of the American Medical Association cited by Theodore and King, in the 1951 and 1953 issues, on the descriptions and uses of cationics one will note the following statement, "Strains of *Pseudomonas aeruginosa* and *Mycobacterium tuberculosis* are particularly resistant to these agents. Bacterial spores remain viable even after prolonged contact with the solutions of the quaternaries." The absence of similar statements in the description and uses of the other chemical preservatives for ophthalmic solutions in official publications does not mean that such chemical compounds are superior in their action against the organisms mentioned. Thus, the inference by Theodore and King that the quaternaries are ineffective is unwarranted and is not supported by this evidence.

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Antispasmodics*

II. α-Amino-α-pyridylacetonitriles and Derivatives

By PAUL JANSSEN, DUSAN ZIVKOVIC, and PAUL DEMOEN

The synthesis of a series of new α-amino-α-pyridyl acetonitriles, -acetamides, and -thioacetamides is reported. Some of these compounds display interesting properties, e. g. tuberculostatic and anticonvulsant activity. Further experiments are in progress.

SINCE A NUMBER of α-amino-α-phenylacetamides, such as serial number A 16, α-dibutylamino-α-(*p*-methoxyphenyl)-acetamide (1, 2, 3) are active musculotropic antispasmodics of low toxicity, it was of interest to prepare α-amino-α-pyridylacetonitriles, -acetamides and -thioacetamides for pharmacological screening.

The 3- and 4-pyridyl-aminonitriles listed in the table, were readily obtained from ice-cooled aqueous solutions of the corresponding pyridine-aldehyde-bisulfite-complexes by adding the amine and an alkali cyanide in molar excess, followed by a variable period of heating, as described in the experimental part.

With 2 - pyridine - aldehyde, however, this method failed to yield the desired nitriles, α-pyridoin being the only newly formed product which could be isolated and identified from the reaction mixture.

The aminoamides were prepared by concentrated sulfuric acid hydrolysis of the nitriles, as previously described for the phenyl-homologues (1).

The thioamides precipitated in quantitative yield from pyridine solutions of the nitriles, containing an aliphatic amine or aminoalcohol (diethylamine, diethanolamine, triethylamine) during a twenty-four-hour saturation period with hydrogen sulfide at room temperature (4).

None of the compounds listed in the table are active antispasmodics. Some of them, however, display other interesting properties, e.g. tuberculostatic and anticonvulsant activity. Further experiments are in progress.

EXPERIMENTAL PART

α - (N - Pyrrolidino) - α - (4 - pyridyl) - acetonitrile (Method A).—A saturated aqueous solution of 11.4 Gm. (0.11 mole) sodium bisulfite was added dropwise to 10.7 Gm. (0.10 mole) isonicotinaldehyde (b. p. 70–72° at 9–10 mm. Hg). After a brief period of stirring the resulting suspension was treated with 14.2 Gm. (0.2 mole) pyrrolidine, followed by 13 Gm. (0.2 mole) of potassium cyanide. Stirring was then continued for several hours at room temperature. By adding two volumes of water, α-(N-pyrrolidino)-α-(4-pyridyl)-acetonitrile separated. It was washed with water and dried (mol. wt. for $C_{11}H_{13}N_3$, calcd.: 187.25; found: 188.0; m. p. 76°; 81% yield).

Similarly prepared were the N-piperidine and N-(4-methyl)-piperidine homologues, listed in the table.

Formation of the bisulfite-adducts at room temperature and subsequent addition of equimolar amounts of the amine gave lower yields.

α-(N-Pyrrolidino)-α-(4-pyridyl)-acetamide.—A mixture of 7.5 Gm. α-(N-pyrrolidino)-α-(4-pyridyl)-acetonitrile and 45 ml. concentrated sulfuric acid was heated for one hour on a water bath, subsequently cooled, and treated with an excess of sodium hydroxide 10 *N*. The precipitate, after recrystallization in chloroform, yielded 84% α-(N-pyrrolidino)-α-(4-pyridyl)-acetamide (mol. wt. for $C_{11}H_{15}N_3O$, calcd.: 205.27; found: ($HClO_4$ titration) 208.0; m. p. 179–180°).

Essentially the same method was adopted for the preparation of the amides listed in the table. The same yields were noted when the reaction mixture was not heated, but kept at room temperature overnight.

α - (N - Pyrrolidino) - α - (4 - pyridyl) - thioacetamide.—A steady stream of hydrogen sulfide was passed through a solution of 5 Gm. α-(N-pyrrolidino)-α-(4-pyridyl)-acetonitrile in 10 ml. pyridine containing 5 ml. diethylamine, triethylamine, diethanolamine or diisopropylamine, at room temperature during a period of twenty-four hours (4). The mixture was then poured into water and the pure thioamide (mol. wt. for $C_{11}H_{15}N_3S$, calcd.: 221.33; found: ($HClO_4$): 221.4; m. p. 169–172° decompn.) collected in quantitative yield by filtration.

This simple method gave equally good yields with the other nitriles listed.

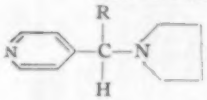
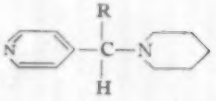
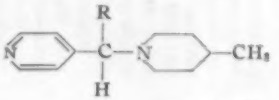
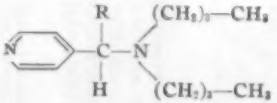
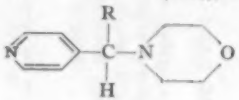
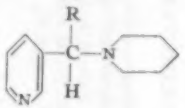
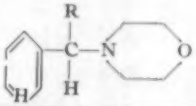
α - (N - di - *n* - butylamino) - α - (4 - pyridyl) - acetonitrile (Method B).—The procedure was essentially the same as the one described above. After addition of the cyanide, the reaction mixture was heated on a steam bath for three hours. The oily nitrile, which separated when water was added, was extracted with ether and purified by distillation *in vacuo* (170° at 8 mm. Hg) (mol. wt. for $C_{18}H_{32}N_3$, calcd.: 245.36; found: 250.0; 69% yield).

The same procedure was adopted for the preparation of the corresponding morpholinonitrile.

Redistilled nicotinaldehyde (b. p. 79–81° at 10 mm. Hg) was used in the synthesis of α-(N-piperidino)-α-(3-pyridyl)-acetonitrile (method A), and of α - (N - morpholino) - α - (3 - pyridyl) - acetonitrile (method B).

* Received March 14, 1955, from the Research Department, Pharmaceutical Laboratories Dr. J. C. Janssen, Turnhout (Belgium).

TABLE I

Basic structure	R	Molecular Formula	Mol. Wt. (a) Calcd. (b) Found ($HClO_4$)	m. p. (m) b. p. (b), °C.	N % (a) Calcd. (b) Found	S % (a) Calcd. (b) Found	% Crude Yield	Solvent of Crystallization	UV-Spectra in i-C_3H_7OH Maxima $m\mu$	Maxima ϵ	Minima $m\mu$	Minima ϵ	Serial No. A
	CN	$C_{11}H_{12}N_2$	(a) 187.25 (b) 188.0	(m) 76	(a) 22.44 (b) 22.1	...	81	H_2O	259.0	2.370	229.0	900	109
	$CONH_2$	$C_{11}H_{14}N_2O$	(a) 205.27 (b) 208.0	(m) 179–180	(a) 20.47 (b) 20.9	...	84	$CHCl_3$	259.5	2.565	241.0	1.925	113
	$CSNH_2$	$C_{11}H_{14}N_2S$	(a) 221.33 (b) 221.4	(m) 169–172 decompn.	(a) 18.99 (b) 19.4	(a) 14.46 (b) 14.6	98	...	264.5 280.0	9.075 8.445	236.0 274.0	3.55 8040	116
	CN	$C_{12}H_{12}N_2$	(a) 201.26 (b) 200.6	(m) 92–94	(a) 20.88 (b) 21.0	...	88	H_2O	259.0	2.375	231.0	945	105
	$CONH_2$	$C_{12}H_{14}N_2O$	(a) 219.29 (b) 220.1	(m) 194–195	(a) 19.86 (b) 20.0	...	79	i-C_3H_7OH	259.5	2.750	240.	2.100	106
	$CSNH_2$	$C_{12}H_{14}N_2S$	(a) 235.36 (b) 237.2	(m) 184–186 decompn.	(a) 17.86 (b) 17.8	(a) 13.60 (b) 13.6	100	...	266.5 280.0	8.945 8.655	237.5 275.5	3075 8365	114
	CN	$C_{18}H_{17}N_2$	(a) 215.29 (b) 218.1	(m) 83–85	(a) 19.52 (b) 19.8	...	77	H_2O	...	...	...	...	236
	$CSNH_2$	$C_{18}H_{19}N_2S$	(a) 249.37 (b) 251.0	(m) 168 decompn.	(a) 16.86 (b) 16.7	(a) 12.84 (b) 12.8	99	...	265.5 280.0	8.900 8.320	236.0 275.0	3298 8240	161
	CN	$C_{15}H_{22}N_2$	(a) 245.36 (b) 250.0	(b) 170/8 mm. Hg	(a) 17.13 (b) 17.0	...	69	...	...	...	...	...	235
	$CONH_2$	$C_{15}H_{24}N_2O$	(a) 263.39 (b) 265.0	(m) 105–106	(a) 17.56 (b) 17.7	...	80	Petrol. ether	258.0	2.595	242.0	2050	110
	CN	$C_{11}H_{12}N_2O$	(a) 203.24 (b) 202.6	(m) 71–72	(a) 20.68 (b) 21.0	...	66	H_2O	259.0	2.290	230.0	655	246
	$CSNH_2$	$C_{11}H_{14}N_2OS$	(a) 237.32 (b) 242.3	(m) 190 decompn.	(a) 17.71 (b) 17.5	(a) 20.23 (b) 20.3	98	...	265.5 281.0	8.274 7.650	237.0 274.0	3164 7405	249
	CN	$C_{12}H_{12}N_2$	(a) 201.26 (b) 204.3	(b) 172/8 mm Hg	(a) 20.88 (b) 20.2	...	73	...	...	...	...	...	234
	$CONH_2$	$C_{12}H_{14}N_2O$	(a) 219.29 (b) 218.9	(m) 148–149	(a) 19.86 (b) 19.1	...	71	Petrol. ether	258.0 262.5	2.770 2.850	240.0 295.5	1920 2720	107
	CN	$C_{11}H_{12}N_2O$	(a) 203.24 (b) 207.4	(b) 180–190/ 7–9 mm. Hg	(a) 20.68 (b) 20.9	...	65	...	...	...	...	...	251
	$CSNH_2$	$C_{11}H_{14}N_2OS$	(a) 237.32 (b) 240.6	(m) 197–198 decompn.	(a) 17.71 (b) 17.3	(a) 20.23 (b) 20.2	99	...	274.0	9.925	241.0	3.250	250

All efforts to prepare the corresponding α-amino-α-(2-pyridyl)-acetonitriles, using method A or B, and picolinaldehyde (b. p. 69–71° at 16 mm. Hg) as the starting material, failed to yield the desired end products. In all these experiments α-pyridoin, orange needles with m. p. 161° (UV spectrum in isopropanol: $\epsilon^{max.}_{347\ m\mu} = 7.850$; $\epsilon^{min.}_{370\ m\mu} = 2.190$; $\epsilon^{max.}_{380\ m\mu} = 18.100$) was the only reaction product which could be isolated and identified.

Anal.—Calcd. for $C_{12}H_{10}N_2O_2$: N, 13.08; mol. wt., 214.22. Found: N, 12.9; mol. wt. ($HClO_4$ titration) 214.2.

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Glucose Ester Derivatives of Aminosalicylic Acids*

By WILLIAM O. FOYE and EDWARD G. FELDMANN†

Glucose esters of 4- and 5-aminosalicylic acid were obtainable by reaction of the benzyloxynitrobenzoic acid chlorides with diacetone glucose, followed by catalytic hydrogenation of the nitro groups. Conversion of the amines to hydrochlorides removed one or both of the acetone groups from the glucose. Complete removal of the acetone groups gave a glucose ester that was unstable on standing, but the protected glucose esters were found to be stable. The free-amino ester of 4-aminosalicylic acid was obtained in excellent yield for tuberculostatic and toxicity determinations.

THE USE of *p*-aminosalicylic acid (PAS) in tuberculosis has several unfavorable aspects, notably the high dosage required, gastrointestinal tract irritation (1), and rather rapid excretion. Although numerous derivatives of PAS have been prepared and examined, none have overcome these disadvantages completely. In an attempt to find derivatives or analogs with more pronounced activity, lower toxicity, and decreased water solubility, PAS derivatives of naturally-occurring compounds have been synthesized. Amides with α-amino acids attached to the carboxyl function were reported previously (2), and this paper describes the formation of glucose derivatives of PAS.

Although conjugation of sugars with phenols and aromatic acids (3) commonly occurs *in vivo*, no definite sugar conjugates of PAS have been reported as excretion products from human subjects fed PAS (4). An observation of the nature and stability of sugar derivatives of PAS should then be of interest. In addition, sugar derivatives of isonicotinyl hydrazide have been shown to improve the properties of this antitubercular agent (5), but little has been done to determine the effect of sugar substituents on the activity of PAS. Haberland (6) has reported the preparation of 4-glycosylaminosalicylic acid derivatives, and Sannié and Lapin (7) have prepared tetraacetylglucose derivatives of PAS, but in neither case were any biological effects discussed.

It has been observed by both Lehmann (8) and Doub (9) that derivatives of PAS involving the carboxyl function, particularly esters, generally retain the antituberculous activity of PAS to a greater extent than the amino- or phenolic-substituted derivatives. Accordingly, esters have been prepared involving the carboxyl group of PAS and a hydroxyl group of the sugar. In order to avoid reactions with the amino and phenol groups of PAS, as well as methods involving preferential hydrolyses, the method of synthesis selected required the use of 4-nitro-2-benzyloxybenzoic acid as starting material, following our experience with the formation of α-amino acid amides of PAS (2). It was also decided to make sugar esters of 5-aminosalicylic acid, since the 5-nitro acid is more readily available for the development of a satisfactory synthesis.

Reaction of the benzyloxynitrobenzoyl chlorides with glucose was found not to occur to any extent in organic solvents and gave too many products in aqueous media to be useful, so the di-isopropylidene derivative (diacetone glucose) was employed. This compound provides a glucose derivative with one free hydroxyl group, with easily removable protective groups, and, being a ketal, should remain stable under alkaline conditions of acylation. Treatment of diacetone glucose with 2-benzyloxy-5-nitrobenzoyl chloride

* Received March 17, 1955, from the School of Pharmacy, University of Wisconsin, Madison.
A portion of this work was abstracted from the thesis presented to the Graduate School of the University of Wisconsin by Edward G. Feldmann as a requirement for the degree of Master of Science, January, 1954.
† Wisconsin Alumni Research Foundation Fellow, 1952–1953; Fellow of the American Foundation for Pharmaceutical Education, 1953–1955.

in benzene pyridine solution produced a gum which was crystallized from chloroform and proved to be a monoacetone derivative of the glucose ester. This was shown by carbon-hydrogen and hydroxyl group analysis, the latter indicating more than one hydroxyl group present, but not more than two. The product did not give reducing sugar tests, and since Freudenberg (10) has reported that the 5,6-isopropylidene hydrolyzes forty times faster than the 1,2-isopropylidene group in glucose, it seemed certain that the acetone was lost from the 5,6-position. It has also been shown by others (11) that in such 3-acyl monoacetone sugar derivatives the acyl group migrates to the free primary hydroxyl at the 6-position. Evidence for this rearrangement was provided by the fact that the melting point underwent gradual change to a constant value during several recrystallizations of this compound from chloroform without change of the elementary analysis.

It is interesting to note that the ester linkage remained stable during washing (with water, dilute acid, and dilute alkali) and recrystallization from chloroform, which is slightly acidic, although one acetone group was completely removed. The ester did not withstand the conditions of the hydroxyl group analysis, however, which required titration with methanolic alkali, since methyl 2-benzyloxy-5-nitrobenzoate was isolated from the reaction in 51 per cent yield.

When the corresponding 4-nitro compound (2-benzyloxy-4-nitrobenzoyl chloride) was treated with diacetone glucose in benzene-pyridine solution, the only product isolated was 2-benzyloxy-4-nitrobenzoic anhydride in 12 per cent yield. A better procedure was accordingly found in the use of the purified acid chlorides in a minimum amount of pyridine as solvent. At zero degrees the pyridine forms a solid with the acid chlorides employed, supposedly the acyl-pyridinium halides, which are powerful acylating agents. Using this procedure, an 83 per cent yield of the diacetone glucose ester of the 5-nitro acid was obtained in less than one hour. A corresponding yield of the 4-nitro ester was also obtained, but the reaction required fourteen hours at room temperature. The melting point of the 4-nitro sugar ester was found to be close to that of methyl 2-benzyloxy-4-nitrobenzoate (methanol was used in the purification of the sugar ester), but an authentic sample of the methyl ester showed them to be nonidentical.

Hydrogenation of the nitro groups was first attempted in ethanol using platinum oxide as catalyst. Only impure products could be obtained, either as free compounds or derivatives, but they did show positive primary amine tests. It should be mentioned that chemical methods of reduction would be unsuitable since acid cleaves the acetone groups as well as the ester linkage, while base was found to cleave these sugar esters rapidly. Using ethyl acetate as solvent, in which the nitro esters are much more soluble than in ethanol, the hydrogenation was found to produce amino esters which could be purified as the hydrochlorides by recrystallization from butanol. Treatment of the amines with hydrogen chloride, however, removed one of the acetone groups from the 5-amino ester and both of the acetones from the 4-amino ester, thus giving a free glucose ester. Both of these isolations were carried out on humid days, but water is also produced during the reduction of the nitro groups, and provides hydrolyzing conditions for the labile ketal groups with the hydrogen chloride present.

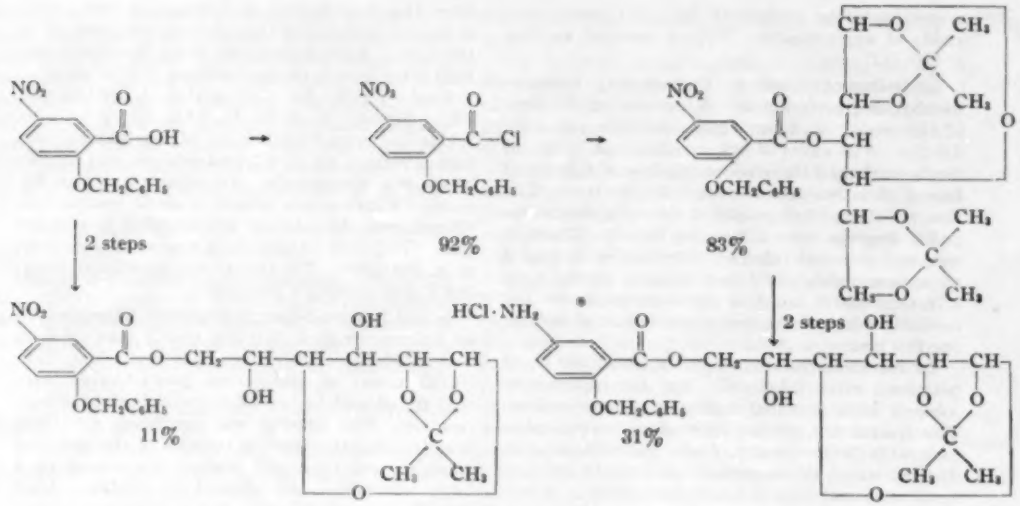
With both acetone groups removed, migration of the acyl group to either the 1- or 6-position is possible. The first possibility is less likely since the compound gave an immediate positive aldehyde test with Benedict's solution, while none of the other acetone-substituted or amino sugars gave a positive test. Since the removal of the acetone groups is a stepwise procedure, and acyl migration to the 6-position immediately follows loss of the 5,6-acetone group, it is highly probable that the free glucose ester is acylated in the 6-position. The glucose also reverts to the pyranose ring after removal of the acetone groups.

The free glucose ester hydrochloride of 2-benzyloxy-4-aminobenzoic acid appeared stable on isolation but decomposed or polymerized to a black residue in somewhat less than two months in a screw-capped vial. In a subsequent preparation of this ester, the amine was not converted to the hydrochloride, and an excellent yield of the diacetone glucose ester was obtained. The reactions leading to sugar ester formations of 2-benzyloxy-5-aminobenzoic acid are shown in Eq. 1, and those of 2-benzyloxy-4-aminobenzoic acid are shown in Eq. 2. The benzyloxy groups were retained in the final products to increase stability and prevent oxidation (2). The biologic activities and toxicities of these compounds will be reported in a later publication.

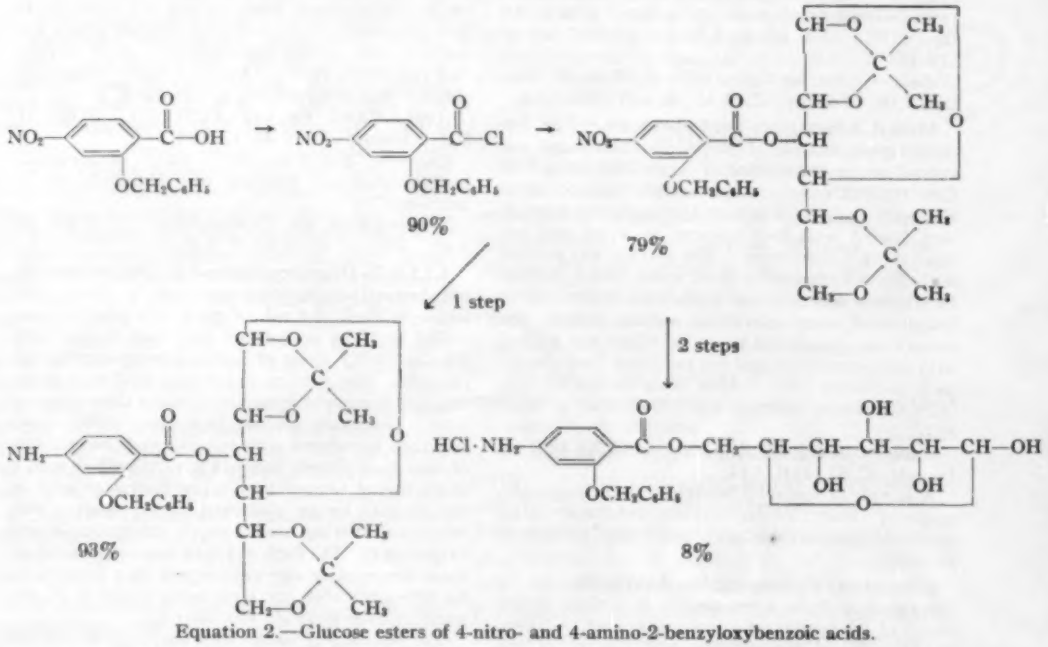
EXPERIMENTAL[1,2]

4-Nitrosalicylic Acid.—This compound was prepared from phenylacetic acid using the method of Borsche (12) as modified by Foye and Hull (2).

[1] All melting points are uncorrected.
[2] The analyses for carbon-hydrogen were done by the Clark Microanalytical Laboratory, Urbana, Ill.



Equation 1.—Glucose esters of 5-nitro- and 5-amino-2-benzyloxybenzoic acids.



Equation 2.—Glucose esters of 4-nitro- and 4-amino-2-benzyloxybenzoic acids.

Over-all yields of 25–30% of cream-colored needles were obtained which melted at 236–238°.

Ethyl 4- and 5-Nitrosalicylate.—Ethyl 4-nitrosalicylate was prepared in 73% yield from 4-nitrosalicylic acid according to the method of Borsche (12); m.p. 86–88°. Ethyl 5-nitrosalicylate was prepared in 93% yield from 5-nitrosalicylic acid (Eastman Organic Chemicals) according to the method of Hirsch (13); m.p. 95–96°. It was found preferable to reflux for six hours instead of three in the latter reaction.

4- and 5-Nitro-2-benzyloxybenzoic Acid.—These acids were prepared by benzylation and hydrolysis of the ethyl nitrosalicylates according to the method of Clinton (14) as modified by Foye and Hull (2). 2-Benzyloxy-4-nitrobenzoic acid was obtained in 85% yield, m.p. 172–174°; and 2-benzyloxy-5-nitrobenzoic acid was obtained in 73% yield, m.p. 165–168°. The melting points agree with the reported values (14, 2).

1,2,5,6-Di-O-isopropylidene-D-glucofuranose (Diacetone Glucose).—This compound was prepared

according to the method of Bell (15), which gave yields of approximately 20% of material melting at 110–111°.

1,2-O-Isopropylidene-6-(2′-benzyloxy-5′-nitrobenzoyl)-D-glucofuranose.—A mixture of 5.0 Gm. (0.018 mole) of 2-benzyloxy-5-nitrobenzoic acid, 4.9 Gm. (0.06 mole) of dry pyridine, and 50 ml. of dry benzene in a three-necked flask was stirred and heated to reflux temperature. At this time, 2.26 Gm. (0.019 mole) of redistilled thionyl chloride was added dropwise from a dropping funnel. The mixture was stirred at reflux for fifteen minutes, cooled to approximately 50°, and treated slowly with 4.76 Gm. (0.018 mole) of diacetone glucose. The resulting solution was stirred and heated at 50° for one-half hour.

To the warm reaction was then added 100 cc. of petroleum ether (Skelly C), and the supernatant solvents were decanted from the gummy residue. This residue was purified by washing several times each with distilled water, dilute hydrochloric acid, distilled water, dilute sodium bicarbonate solution, again with water, and finally with ether. It was then taken up in a minimum quantity of acetone, decanted from a bit of insoluble material, and evaporated to dryness. The resulting material was twice recrystallized from chloroform, giving 1.0 Gm. (11%) of a microcrystalline product, m. p. 172–173°.

Anal.—Calcd. for $C_{23}H_{25}O_{10}N$: C, 58.09; H, 5.30; OH, 7.16. Found: C, 57.45; H, 5.37; OH, 5.18.

Methyl 2-Benzyloxy-5-nitrobenzoate.—The hydroxyl group analysis of the previous compound was carried out by the method of Siggia (16), using 0.66 Gm. (0.0014 mole). After acetylation and titration with methanolic sodium hydroxide, the solution was diluted with two volumes of water and extracted with chloroform. The extract was washed with dilute hydrochloric acid, water, dilute sodium bicarbonate solution, and again with water. After being dried over anhydrous sodium sulfate, the extract was evaporated to a solid which was washed with petroleum ether and recrystallized from chloroform-petroleum ether. After being chilled, 0.2 Gm. (51%) of shining platelets was collected, m. p. 88.5–89.5°.

Anal.—Calcd. for $C_{15}H_{13}O_5N$: C, 62.70; H, 4.56. Found: C, 63.11; H, 4.74.

A sample of methyl 2-benzyloxy-5-nitrobenzoate, prepared from 2-benzyloxy-5-nitrobenzoic acid, methanol, and sulfuric acid, was found to melt at 88–90°.

2-Benzyloxy-4-nitrobenzoic Anhydride.—In a three-necked flask were placed 10.0 Gm. (0.037 mole) of 2-benzyloxy-4-nitrobenzoic acid, 9.7 Gm. (0.118 mole) of dry pyridine, and 100 ml. of dry benzene. The mixture was stirred and heated to reflux, when 4.5 Gm. (0.038 mole) of thionyl chloride was introduced dropwise. The mixture was then refluxed with stirring for fifteen minutes, cooled to approximately 45°, and treated with 9.5 Gm. (0.037 mole) of diacetone glucose. Heating at 45° was maintained for one-half hour longer.

The solvent was decanted from the gummy residue (from which no product was isolated) and was washed with dilute hydrochloric acid, water, dilute sodium bicarbonate solution, and water. After dilution with three volumes of petroleum ether and chilling for two days, crystal formation started. After two days further of chilling, 1.2 Gm. (12%) of slender, rod-shaped crystals were collected, m. p. 151–153°. Recrystallization from benzene-petroleum ether gave a product melting at 153–153.5°.

Anal.—Calcd. for $C_{28}H_{29}O_9N_3$: C, 63.63; H, 3.82. Found: C, 63.84; H, 3.88.

One-half gram (0.001 mole) of this material was heated with 10 ml. of 5% hydrochloric acid for two hours on a steam bath. An excess of sodium hydroxide solution was added, a small residue was filtered, and the solution was acidified to give 0.4 Gm. (77%) of 2-benzyloxy-4-nitrobenzoic acid, m. p. 166–169°. The filtrate was negative to Benedict's and Fehling's solutions.

4- and 5-Nitro-2-benzyloxybenzoyl Chloride.—In an Erlenmeyer flask 20.0 Gm. (0.073 mole) of 4- or 5-nitro-2-benzyloxybenzoic acid and 40.0 Gm. (0.192 mole) of phosphorus pentachloride were well mixed and heated on a steam bath to induce reaction. The heating was continued for three minutes after the vigorous reaction of the melt had subsided, and the liquid product was poured on a large watchglass and allowed to solidify. After solidification was complete, the material was rubbed vigorously on a clay plate with a spatula to remove phosphorus oxychloride. The resulting powder was twice recrystallized from carbon tetrachloride to give glistening platelets of acid chloride which was washed with carbon tetrachloride. After drying, 19.2 Gm. (90% yield) of 4-nitro-2-benzyloxybenzoyl chloride was obtained, m. p. 123–124.5°.

Anal.—Calcd. for $C_{14}H_{10}O_4NCl$: C, 57.65; H, 3.46. Found: C, 57.44; H, 3.34.

Likewise, 19.6 Gm. (92% yield) of 5-nitro-2-benzyloxybenzoyl chloride was obtained, m. p. 103.5–104.5°.

Anal.—Calcd. for $C_{14}H_{10}O_4NCl$: C, 57.65; H, 3.46. Found: C, 57.67; H, 3.42.

1,2,5,6-Di-O-isopropylidene-3-(2′-benzoyloxy-5′-nitrobenzoyl)-D-glucofuranose.—In a 25-cc. Erlenmeyer flask, 6.0 ml. of pure, dry pyridine was cooled to 0° by an ice-bath and then treated with 2.0 Gm. (0.007 mole) of 5-nitro-2-benzyloxybenzoyl chloride. The mixture was stirred until homogeneous, and it completely solidified about three minutes later. Diacetone glucose (1.8 Gm., 0.007 mole) was then introduced with stirring, and the contents of the flask almost immediately reliquified with a sharp rise of temperature. The flask was kept in the ice bath for an additional fifteen minutes with occasional stirring, and crystals gradually formed in quantity. The flask was then allowed to warm to room temperature and was warmed on a steam bath for fifteen minutes, and after being cooled to 0°, the pyridine hydrochloride was filtered.

The filtrate was poured into ten times its volume of absolute methanol, and a fine, white powder separated within a few minutes. After recrystallization from absolute methanol, 2.9 Gm. (83%) of glistening clusters of fine needles were collected, m. p. 135.5–137°.

Anal.—Calcd. for $C_{29}H_{29}O_{10}N$: C, 60.59; H, 5.67. Found: C, 60.86; H, 5.57.

1,2,5,6-Di-O-isopropylidene-3-(2′-benzyloxy-4′-nitrobenzoyl)-D-glucofuranose.—In a 25-cc. Erlenmeyer flask were placed 2.0 Gm. (0.007 mole) of 2-benzyloxy-4-nitrobenzoyl chloride and 1.8 Gm. (0.007 mole) of diacetone glucose. The solids were thoroughly mixed, and 6.0 ml. of dry pyridine was

introduced. The contents of the flask warmed in a short time, and crystals began to separate. After standing for fourteen hours at room temperature, the reaction mixture had solidified, and it was then poured into 60 ml. of methanol containing 0.5 ml. of water. A gummy product separated immediately, and the supernatant liquid was decanted. The gum was crystallized from absolute methanol to give 2.8 Gm. (79%) of fine needles, m. p. 99.5–101°.

Anal.—Calcd. for $C_{26}H_{29}O_{10}N$: C, 60.59; H, 5.67. Found: C, 60.28; H, 5.38.

Methyl 2-Benzyloxy-4-nitrobenzoate.—From 2.0 Gm. (0.007 mole) of 2-benzyloxy-4-nitrobenzoic acid, 20 ml. of methanol, and 2 ml. of concentrated sulfuric acid was obtained 1.4 Gm. (66%) of silken needles of ester after recrystallization from methanol, m. p. 90–91°.

Anal.—Calcd. for $C_{15}H_{13}O_5N$: C, 62.70; H, 4.56. Found: C, 62.88; H, 4.35.

A mixed melting point with 1,2,5,6-di-O-isopropylidene-3-(2′-benzyloxy-4′-nitrobenzoyl)-D-glucofuranose was depressed to 72–77°.

1,2-O-Isopropylidene-6-(2′-benzyloxy-5′-aminobenzoyl)-D-glucofuranose Hydrochloride.—In a 500-cc. hydrogenation bottle, 5.2 Gm. (0.010 mole) of 1,2,5,6-di-O-isopropylidene-3-(2′-benzyloxy-5′-nitrobenzoyl)-D-glucofuranose was dissolved in 80 ml. of anhydrous ethyl acetate. Adams' platinum oxide catalyst (0.1 Gm.) was then added, and the mixture was hydrogenated at 35 p. s. i. After thirty minutes, the hydrogen uptake was complete, and the solution was filtered through quantitative filter paper to remove catalyst. The ethyl acetate was evaporated under reduced pressure at 30°, the evaporation was repeated several times with ether, and the resulting syrup was dissolved in ether and saturated with dry hydrogen chloride. After being chilled, the precipitate of hydrochloride was filtered and washed with ether, but became gummy during this operation.

After being dried in a desiccator, the solidified material was pulverized and heated in butanol. Minute needles separated and were washed several times each with butanol and ether. The yield was 1.5 Gm. (31%) of crystalline material which melted at 182–183° with decomposition. The compound was soluble in water, but gave an immediate precipitate on addition of alkali; it gave positive primary amine tests with nickel chloride-5-nitrosalicyl aldehyde and nitrous acid.

Anal.—Calcd. for $C_{23}H_{28}O_8NCl$: C, 57.31; H, 5.86. Found: C, 57.58; H, 5.72.

6-(2′-Benzyloxy-4′-aminobenzoyl)-D-glucopyranose Hydrochloride.—This compound was obtained by using the preceding hydrogenation procedure on 3.0 Gm. (0.006 mole) of 1,2,5,6-di-O-isopropylidene-3-(2′-benzyloxy-4′-nitrobenzoyl)-D-glucofuranose in 40 ml. of anhydrous ethyl acetate. After conversion of the reduced product to the hydrochloride in ether, the resulting gum was dried and powdered to give 2.5 Gm. of material which melted at 104–115° with decomposition. The ether filtrate, however, was concentrated to one-half its volume and chilled to give 0.2 Gm. (8%) of material which melted at 116–119° with decomposition, after washing with ether, drying, and pulverizing. This fraction was analyzed.

Anal.—Calcd. for $C_{20}H_{24}O_8NCl$: C, 54.36; H, 5.47; OH, 15.4. Found: C, 53.86; H, 5.56; OH, 13.7.

The hydroxyl group analysis was carried out by the method of Siggia (16). Both fractions strongly reduced Benedict's solution, indicating that the first fraction also contained an appreciable amount of the free glucose ester.

1,2,5,6-Di-O-isopropylidene-3-(2′-benzyloxy-4′-aminobenzoyl)-D-glucofuranose.—The same hydrogenation procedure as in the preceding cases was used to obtain this compound, starting with 5.2 Gm. (0.010 mole) of 1,2,5,6-di-O-isopropylidene-3-(2′-benzyloxy-4′-nitrobenzoyl)-D-glucofuranose in 80 ml. of anhydrous ethyl acetate. A Celite mat (prepared from Celite suspended in ethyl acetate) was used to filter out the catalyst, however. The gummy product resulting from complete evaporation of the ethyl acetate was dissolved in 20 ml. of dry ether, and petroleum ether (b. p. 60–68°) was introduced until precipitation of the gum was complete (about 200 ml. were required). The resulting mixture was placed in an acetone dry-ice bath for an hour, and the solidified product was rapidly filtered and washed with cold petroleum ether. After being dried in high vacuum for five hours, the amorphous solid was ground to a fine powder. A yield of 4.5 Gm. (93%) was obtained which melted at 70–74°.

Anal.—Calcd. for $C_{26}H_{31}O_8N$: C, 64.34; H, 6.44. Found: C, 65.10; H, 6.85.

SUMMARY

1. Glucose esters of 4- and 5-nitrosalicylic acids have been prepared from diacetone glucose and the nitro-O-benzylsalicyloyl chlorides. These compounds were isolated as the O-benzyl diacetone derivatives and were readily converted to the 4- and 5-amino esters on hydrogenation. Conversion of the amines to hydrochlorides removed one or both acetone groups. A glucose ester with no acetone groups remaining proved to be unstable on standing.

2. The following new compounds were characterized: 4- and 5-nitro-2-benzyloxybenzoyl chloride, methyl 4- and 5-nitro-2-benzyloxybenzoate, 2-benzyloxy-4-nitrobenzoic anhydride, 1,2-O-isopropylidene-6-(2′-benzyloxy-5′-nitrobenzoyl)-D-glucofuranose, 1,2,5,6-di-O-isopropylidine-3-(2′-benzyloxy-4′ (and 5′)-nitrobenzoyl)-D-glucofuranose, 1, 2-O-isopropylidene-6-(2′-benzyloxy-5′-aminobenzoyl)-D-glucofuranose hydrochloride, 6-(2′-benzyloxy-4′-aminobenzoyl)-D-glucopyranose hydrochloride, and 1,2,5,6-di-O-isopropylidene-3-(2′-benzyloxy-4′-aminobenzoyl)-D-glucofuranose.

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A Study of Sublingual Absorption II*

Striated Muscle Relaxants and Neurovegetative Blocking Agents

By MARTIN KATZ† and MARTIN BARR

Solutions of drugs were sublingually administered to anesthetized animals whose esophagus had been ligated and trachea cannulated. This method was devised so that there would be no possibility of the drug being swallowed. In comparison with effective intravenous doses, very high sublingual doses of *d*-tubocurarine chloride, Metubine® iodide, Anectine®, and Flaxedil® were ineffective in blocking the response of the gastrocnemius muscle to sciatic stimulation. The sublingual administration of from three to six times the intravenous dose of Dibenamine® hydrochloride or ten times the intravenous dose of Hydergine® produced a slow, mild adrenergic blockade as measured by the response to epinephrine of anesthetized dogs.

THIS INVESTIGATION was undertaken to ascertain whether the sublingual route of administration was feasible for several drugs which have been used mostly by the parenteral route or which are subject to irregular oral absorption. A survey of the literature related to sublingual absorption has been presented and a method of study described (1).

CURARE AND CURARE-LIKE ALKALOIDS

Curare and the curare-like alkaloids have found increasing use in neurology and surgery (2). However, their ineffectiveness by the oral route has been a drawback to their more widespread use, limiting their administration to the intravenous and intramuscular routes (3–5).

Delay, *et al.* (6, 7), obtained interesting results by the use of rectal suppositories of *d*-tubocurarine chloride. Erdei (8) and Neff and Mayer (9) used sublingual tablets of *d*-tubocurarine.[1,2] In a therapeutic dosage of 6–12 mg., they were found to be nontoxic, rapid acting, and very effective in relieving pain due to acute and chronic muscle spasm.

The relative ineffectiveness of oral curare has been explained on the basis of its slow absorption in the alimentary canal and its rapid elimination in the urine. The liver is believed to detoxify curare, although it is probably not the only site (10, 11). It is thought that circumventing the liver may be in part responsible for the efficiency of the sublingual and rectal administration of curare.

Experimental.—For an evaluation of curariform activity by sublingual administration, the intact gastrocnemius-sciatic nerve technique was used (12, 13). Since premedication of animals with a barbiturate increases the sensitivity to *d*-tubocurarine chloride while urethane does not (14), the animals were anesthetized with an intraperitoneal injection of urethane 1.2 Gm./Kg. They were operated on in the manner previously described (1), having the esophagus ligated and the trachea cannulated. Since it is well known that the curariform drugs cause respiratory depression, the tracheal cannula was connected to a respiratory pump. One jugular vein was cannulated to provide a means for intravenous injection. The animal was fixed on a board over a heating pad and its legs were immobilized. The sciatic nerve was isolated and cut centrally. This nerve received stimulation through the electrodes of an inductorium, operated to deliver from 40–50 stimuli per minute (15, 16) by a Harvard timer run by a battery eliminator delivering a con-

* Received February 15, 1955, from the Philadelphia College of Pharmacy and Science, Philadelphia, Pa.
Presented to the Scientific Section, A. PH. A., Boston meeting, August, 1954.
Abstracted in part from a study conducted in the Department of Pharmacy and the Department of Pharmacology and submitted as a dissertation to the Graduate School of the Philadelphia College of Pharmacy and Science in partial fulfillment of the requirements for the degree of Doctor of Science.
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The authors wish gratefully to acknowledge the excellent suggestions and criticisms of Dr. Julian Ambrus, Professor of Pharmacology. We are also indebted to Dr. J. W. E. Harrisson for placing the facilities of the LaWall and Harrisson Laboratories, Philadelphia, Pa., at our disposal.

[1] Brent Laboratories, Toronto, Canada.
[2] Tubalex,® Cugil Laboratories, Palo Alto, Calif.

stant six-volt supply. The nerve and muscle were kept moist with Locke-Ringer's solution kept at 37°. The gastrocnemius muscle was prepared, exposing as little of it as possible. A sheaf from the tendon was attached to a myograph which registered the contractions provoked by the excitation of the sciatic nerve on a kymograph.

One dog, three rabbits, and 20 rats were used in this study. The drugs were used sublingually in doses ranging from 20–500 times the injection dose. After sublingual administration of these large doses, muscle response was observed for fifteen to thirty minutes. When no blocking was observed sublingually, the smaller, effective parenteral dose was administered intravenously as proof of muscle sensitivity and the experimental method. After effective blocking of muscle response had been obtained with an intravenous injection of *d*-tubocurarine chloride, Metubine® iodide, or Flaxedil®, the curare antagonist, Tensilon® chloride (17), 10 mg./cc.,[3] was employed as further proof that the blocking of the muscle response was due to the curariform drug.

Several curariform drugs were tested in this manner. Table I lists these drugs, their manufacturer, the concentration of the available solution, the effective dose by intravenous injection and reference, and the sublingual doses which were used, ranging from 20–500 times the injection dose. Each of the curariform drugs was also administered sublingually with 10 turbidity reducing units of hyaluronidase. Normally, 150 turbidity reducing units are used in the hypodermoclysis of 1000 cc. of solution (21). The curariform drugs in the doses listed above were also administered sublingually with 0.05 cc. of a mixture containing 50% each of Span® 80 and Tween® 80.[5]

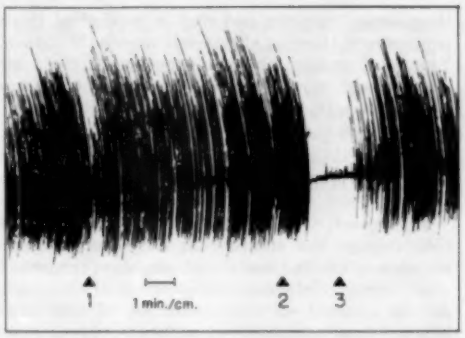

Fig. 1.—Sublingual and intravenous administration of *d*-tubocurarine chloride.—(1) 15 mg./Kg. s. l.; (2) 0.15 mg./Kg. i. v.; (3) Tensilon® chloride 25 mg./Kg. i. v.

Discussion.—In this study of the sublingual absorption of curare and the curare-like alkaloids, from 20–500 times the parenteral dose failed to exert any effect sublingually.

Some explanation for this discrepancy between experimental and clinical findings may be found in the paper by Neff and Mayer (9) on the use of sublingual curare. They have observed that sublingual pellets or rectal suppositories elicit only a sub-subjective infracurarization. Overdosage with these dosage forms of as much as 78 times the lethal dose failed to cause any observable signs of curarization in any animal. When the drug was taken sublingually up to 54 mg. daily (but in the absence of any clinical lesion) no subjective effect whatsoever was noted. There were no signs of cumulative action and no toxic or unpleasant side effects were observed. Blood level determinations, sensitive to 2 mg./cc., were unable to detect any curare. "It would appear that spastic muscles are much less resistant to curare

TABLE I.—CURARIFORM DRUGS TESTED

Drug	Source	mg./cc.	I. V. Dose, mg./Kg.	Ref.	S. L. Dose, mg./Kg.
d-Tubocurarine chloride	U. S. P. XIV[4]	15.0	0.05	(13)	15.0
Metubine® iodide	Lilly	0.5	0.01	(13)	0.5
Anectine® (succinylcholine)	Burroughs Wellcome	50.0	0.1	(18)	3.5–50.0
Syncurine® (decamethonium)	Burroughs Wellcome	1.0	0.03	(19)	1.0
Flaxedil®	Lederle	20.0	1.0	(20)	20.0

Results.—In sublingual doses of from 20–500 times the demonstrated effective parenteral dose, none of the five drugs tested showed any evidence of producing even a partial blockade of muscle response to sciatic nerve stimulation. Figures 1 and 2 are typical examples of the experiments performed. In both cases, the sublingual administration of 100 times the parenteral dose to rats failed to exert any effect while the intravenous injection produced immediate muscle relaxation. In Fig. 1, Tensilon® chloride, 25 mg./Kg., was used to antagonize the action of intravenous *d*-tubocurarine chloride. In Fig. 2, the relaxation produced by the intravenous administration of the ultra-short-acting Anectine® (succinylcholine) rapidly disappears.

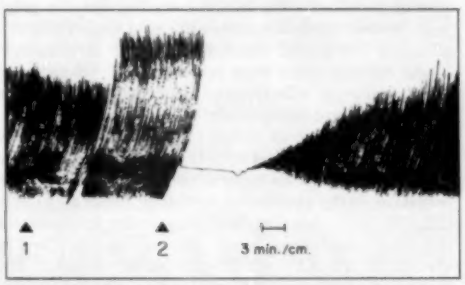

Fig. 2.—Sublingual and intravenous administration of Anectine® (succinylcholine)—(1) 50 mg./Kg. s. l.; (2) 0.1 mg./Kg., i. v.

[3] The courtesy of Hoffmann-LaRoche, Nutley, N. J., in providing the Tensilon chloride used in this study is gratefully acknowledged.

[4] We wish to express our thanks to Abbott Laboratories, No. Chicago, Ill., and E. R. Squibb and Sons, New York, N. Y., for providing us with the *d*-tubocurarine chloride used in this study.

[5] Atlas Powder Co., Wilmington, Del.

than normal muscles and that in subliminal therapeutic doses, the drug affects only muscles in spasm." This may explain why our experiments could not demonstrate any sublingual absorption of curare, while others (8, 9) have found it effective for the relief of muscle spasm.

NEUROVEGETATIVE BLOCKING AGENTS

Hydergine®.—The work of Rothlin (22) and his collaborators has shown that dihydroergocornine, dihydroergocristine, and dihydroergokryptine, which when combined in equal parts constitute Hydergine®,[6] are all effective in the production of adrenergic blockade and peripheral vasodilation. Hydergine® injection has been found to be an effective agent for the treatment of hypertension and peripheral vascular diseases (23, 24).

One of the actions of Hydergine® which helps improve peripheral circulation is its ability to block adrenergic stimuli. Hydergine® blocks transmission at the neuro-effector junction and inhibits the effects of injected epinephrine. In anesthetized intact dogs, complete inhibition or reversal of the pressor effect of from 0.005 to 0.02 mg. of epinephrine was obtained in from fifteen to thirty minutes with the injection of from 0.05 to 0.2 mg./Kg. of Hydergine® (22, 23). Orth (25) found that in some dogs, the blood pressure rise became smaller with no subsequent fall evident, while in others a subsequent fall in blood pressure was seen but only after an initial brief but definite rise in response to epinephrine.

Since Hydergine® is poorly absorbed orally, its use had been mostly limited to parenteral administration. The many uses for this compound warranted an investigation to determine whether it was absorbed sublingually. A study of the blood pressure response to epinephrine injections after Hydergine® was undertaken. While these studies were in progress, several clinical reports appeared concerning the effective sublingual administration of Hydergine®. These articles have been reviewed (1). Because of their apparent effectiveness, sublingual tablets of Hydergine® are being made commercially available.

Experimental.—In order to study the sublingual absorption of Hydergine®, the effect on the blood pressure response to epinephrine injections was observed as in the studies by Rothlin, Goetz, and Orth, (22, 23, 25). Anesthetized dogs were prepared for the direct reading of blood pressure as described in the U. S. P. XIV (26) epinephrine assay. The trachea was cannulated and the esophagus ligated so that solutions could be administered sublingually.

The vehicle of the solution for sublingual administration consisted of equal parts of water, alcohol, and propylene glycol. The solutions for intravenous injection were aqueous. Eight dogs were used in this study. Typical portions of several kymograph recordings are presented in Figs. 3 and 4.

Results.—The pattern or response which would signify the appearance of adrenergic blockade after absorption of Hydergine® was demonstrated in Fig. 3 by the lessening of the blood pressure response to epinephrine after the intravenous injection of 0.15 mg./Kg. of Hydergine®. After forty minutes, the response to epinephrine consisted of an initial brief but definite rise followed by a subsequent fall in blood pressure. This was similar to the pattern described by Orth (25). As seen in Fig. 4, an hour after the sublingual administration of 3 mg./Kg. of Hydergine®, the same response pattern to epinephrine was observed as after the intravenous administration of Hydergine® (Fig. 3). The similar initial brief rise followed by the gradual drop and subsequent rise to normal indicated that Hydergine® was absorbed sublingually.

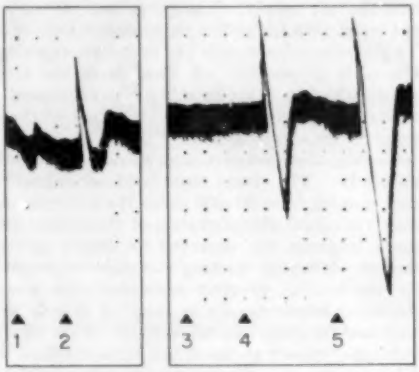

Fig. 3.—Intravenous Hydergine®.—(1) 0 min., Hydergine®, 0.075 mg./Kg. i. v.; (2) 5 min., Epi., HCl, 1.25 μg./Kg.; (3) 30 min., Hydergine®, 0.075 mg./Kg. i. v.; (4) 35 min., Epi. HCl, 1.25 μg./Kg.; (5) 40 min., Epi. HCl, 3.75 μg./Kg.

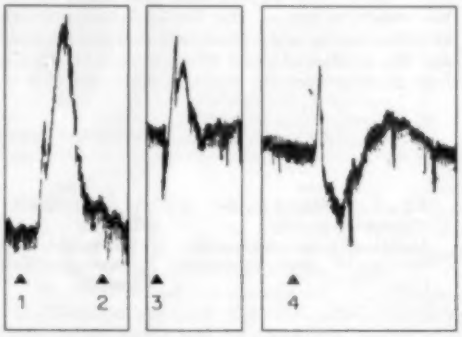

Fig. 4.—Sublingual Hydergine®.—(1) Epi. HCl, 5 μg./Kg.; (2) 0 min., Hydergine®, 3.0 mg./Kg. s. l.; (3) 30 min.; Epi. HCl, 5 μg./Kg.; (4) 75 min., Epi. HCl, 5 μg./Kg.

Discussion.—From the experiments performed, it was seen that the sublingual administration of about ten or more times the intravenous dose reduced the blood pressure response to epinephrine to about one-half or one-third in two hours. The response pattern of adrenergic blockade, an initial brief rise followed by a larger gradual blood pressure drop, then appeared. This pattern was noted within fifteen minutes after the intravenous administration of Hydergine® and was also seen in the work by Orth (25).

The sublingual absorption of Hydergine® in these experiments seemed much slower and milder than the ratio of 1:4 which is indicated in the clinical re-

[6] We wish to thank Sandoz Pharmaceuticals, New York, N. Y., for providing the Hydergine® used in this study.

ports on sublingual Hydergine® (1). However, these reports compared sublingual absorption to intramuscular not intravenous administration.

Dibenamine®.—In anesthetized animals, the slow intravenous injection of 10–20 mg./Kg. of the sympatholytic agent, Dibenamine®[7] will completely reverse the blood pressure effect of such massive intravenous injections of epinephrine as 1 mg./Kg. Its effect starts in ten minutes, becomes maximal in from thirty to ninety minutes, and lasts one to five days (27–29). Blocking is usually complete and irreversible and probably results in destruction of the receptors. When the action is wearing off, partial blocking may be observed. Small doses of epinephrine will then effect a brief pressor action followed by a depressor phase while larger doses will give an almost pure pressor response.

Dibenamine® produces local tissue damage and also causes central nervous system stimulation. Rapid intravenous injection will elicit coordinated clonic convulsions. Since the toxic dose varies with the route and speed of administration, proper selection of these is very important. The untoward side reactions and the capricious nature of the response to Dibenamine® when given intramuscularly, subcutaneously, intraperitoneally, and orally have seriously hampered its widespread clinical use. For these reasons, the sublingual route of administration was considered for Dibenamine®.

Experimental.—A 10% solution of Dibenamine® hydrochloride in propylene glycol was prepared and administered sublingually to four anesthetized dogs prepared for the direct reading of blood pressure as described in the U. S. P. XIV (26) epinephrine assay. The trachea was cannulated and the esophagus ligated so that solutions could be administered sublingually. Comparisons were made with intravenous injections of Dibenamine®. The responses to intravenous epinephrine were observed for signs of adrenergic blockade. Portions of typical kymograph recordings are reproduced in Figs. 5 and 6.

Fig. 5.—Intravenous Dibenamine®.—(1) Epinephrine HCl 3μg./Kg., i. v.; (2) Epinephrine HCl, 9 μg./Kg. i. v.; 35 minutes after Dibenamine® HCl 20 mg./Kg., i. v.

[7] We gratefully acknowledge the courtesy of the Smith, Kline & French Laboratories of Philadelphia, Pa., in providing the Dibenamine® used in this study.

Results.—After the intravenous administration of 10–20 mg./Kg. of Dibenamine® hydrochloride, the pressor response to epinephrine was rapidly lessened. Within 30–60 minutes, the response to epinephrine was characterized by a small fleeting rise followed by a large, longer lasting fall and slow return to normal (Fig. 5). With sublingual doses of 50–100 mg./Kg., a similar pattern, with a less pronounced reversal of epinephrine response, was observed in about four hours (Fig. 6).

Discussion.—The experiments which were performed indicated that Dibenamine® hydrochloride was absorbed when administered sublingually. From three to six times the intravenous dose provided slow, mild action. Divided doses of a solution were more readily absorbed than a large dose of a suspension.

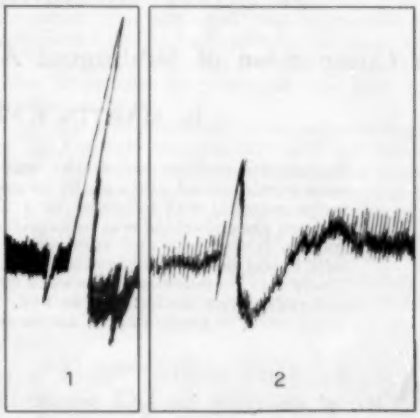

Fig. 6.—Sublingual Dibenamine®.—(1) Epinephrine HCl 20 μg./Kg. i. v.; (2) Epinephrine HCl 40 μg./Kg., i. v., 225 minutes after Dibenamine® HCl 100 mg./Kg. s. l.

SUMMARY

1. Solutions of drugs were sublingually administered to anesthetized animals whose esophagus had been ligated and trachea cannulated.

2. In comparison with effective intravenous doses, very high sublingual doses of *d*-tubocurarine chloride, Metubine® iodide, Anectine®, and Flaxedil® were ineffective in blocking the response of the gastrocnemius muscle to sciatic stimulation.

3. The sublingual administration of from three to six times the intravenous dose of Dibenamine® hydrochloride or ten times the intravenous dose of Hydergine® produced slow, mild adrenergic blockade as measured by the response to epinephrine.

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A Study of Sublingual Absorption III*

A Comparison of Sublingual Absorption from Various Tablet Bases

By MARTIN KATZ† and MARTIN BARR

Radioactive sodium iodide-131 was incorporated into twelve tablet bases which were administered sublingually to anesthetized mice. The absorption of the radioactive material was followed by a Geiger-Muller counter tube held over the tail. Sodium phenobarbital was incorporated into the same tablet bases as the radioactive sodium iodide-131 and administered sublingually to anesthetized rabbits. Periodic blood level determinations were made with the ultraviolet spectrophotometer. There was no correlation between the rates of absorption of sodium phenobarbital and radioactive sodium iodide-131. The rates of *in vitro* or *in vivo* tablet disintegration could not be related to the rates of absorption.

A SURVEY of the literature related to sublingual absorption has been presented (1) and additional studies have been performed (2). This investigation was undertaken to ascertain the efficacy of various tablet bases for sublingual absorption. Much work has been done on the determination of the rates of absorption of substances from different suppository and ointment bases (3, 4). Many substances have been proposed for comparing the efficacy of the different bases used and *in vivo* and *in vitro* methods have been devised for comparing rates of liberation or absorption. For this study of "oral suppositories," bases were chosen from the groups used in the suppository study of Gross and Becker (5). Radioactive sodium iodide-131 and sodium phenobarbital were used to compare the rates of absorption from the sublingual route of administration.

* Received February 15, 1955, from the Philadelphia College of Pharmacy and Science, Philadelphia, Pa.
Presented to the Scientific Section, A. PH. A., Boston meeting, August, 1954.
Abstracted in part from a study conducted in the Department of Pharmacy and the Department of Pharmacology, and submitted as a dissertation to the Graduate School of the Philadelphia College of Pharmacy and Science in partial fulfillment of the requirements for the degree of Doctor of Science.
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The authors wish to acknowledge the valuable suggestions and criticisms of Dr. Julian Ambrus and Dr. Arthur Osol.

EXPERIMENTAL

Radioactive Sodium Iodide-131.—Radioactive isotopes have been found to be effective for labeling and tracing stable nonradioactive isotopes. Minute traces of a labeled compound, which have been absorbed, can be detected even if the stable compound is normally present in the body.

Several pharmaceutical problems, such as the study of enteric coatings (6, 7), ointments (8–10), suppositories (11), and a comparison of routes of absorption (12), have made use of the unique properties of radioisotopes.

Sodium Iodide-131 Solution.—The sodium iodide was obtained from Oak Ridge[1] as a solution containing 10 mc. of sodium iodide-131 in 0.9 cc. of 0.1% sodium bisulfite solution, *p*H 8.0.

Preparation of Tablets.—The tablet bases used are listed in Table I. The lactose and sucrose tablet triturates were prepared according to the pharmaceutical art (13). The others were prepared by the fusion process commonly used for suppositories (14). A one-quarter grain (16 mg.) tablet triturate mold,[2] was used in both cases. Each tablet was made to contain 1 μc. of radioactive sodium iodide-131 and 1% of sodium iodide.

Standardization of Tablets.—The tablets of a group were individually weighed and a count of each was taken with a G-M tube. The variations

[1] United States Atomic Energy Commission, Oak Ridge, Tenn.
[2] Our most sincere thanks to Dr. Paul Wilcox of Sharp and Dohme, Inc., West Point, Pa., for his kindness in making this mold available.

were well within ±5%. Tablets from each group were also dissolved or dispersed in water and aliquots were removed for an assay of radioactivity. The actual potencies of the various tablet batches conformed to the theoretical potencies well within a range of ±5%.

Method of Study.—A very convenient method for tracing certain radioactive materials, such as I-131, P-32, and Au-198, in the circulation of mice was developed in our laboratories (15). A special lead shield was cast which provides a shielded groove for the mouse tail over which a shielded G-M

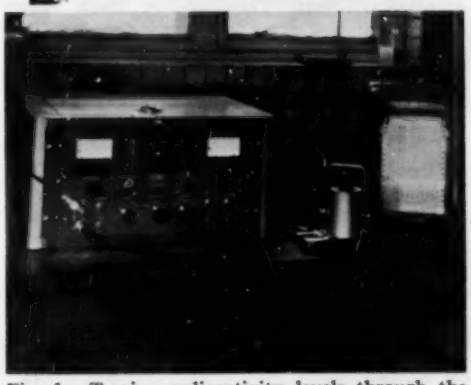

Fig. 1.—Tracing radioactivity levels through the tail surface of a mouse.

tube[3] is placed (Fig. 1). In this way the background count has been greatly reduced providing greater sensitivity. The use of mice is advantageous in several ways. They are less expensive and their size permits the use of smaller quantities of the relatively expensive radioisotopes. Of even greater importance is the thinness of the tail of the mouse as compared to that of the rat. This permits the detection of less powerful emitters as well as smaller concentrations of radioisotopes.

[3] El Tronics, Shielded Counter, Philadelphia, Pa.

The G-M tube is connected to a counting rate meter[4] which records counts per second and a Scaler[5] which records total counts. The readings on the rate meter are recorded with a graphic ammeter.[6] This permits simultaneous recording of total counts and counts per second. These counts are proportional to the concentration of the radioisotopes in the circulation. With this arrangement the rate of disappearance from the circulation of several intravenously injected radioactive materials has been successfully studied (16). It can also be used for a study of the absorption into the circulation of a radioisotope administered parenterally, orally, sublingually, rectally, and externally.

Administration of Tablets.—For this study the sublingual tablets containing radioactive sodium iodide-131 were administered sublingually to mice anesthetized with urethane, 1.25 Gm./Kg., in which the esophagus had been ligated and the trachea cannulated. The sublingual solution or tablet was administered after a background count was taken and the counts per second from the tail were recorded at periodic intervals. At least four mice were used per group.

This data was statistically evaluated according to the small sample method (17) and an average hourly value determined for each group. The average rates of absorption and the standard errors of the hourly means as determined for all the tablet bases used in this study are presented in Table I. In order to evaluate more readily the results, the counts/sec. at three hours were used to develop an arbitrary index for comparison, with the highest base equated to 100 and the other bases scaled accordingly.

Rates of Disintegration.—*In Vitro.*—Samples of each tablet were placed in 50 cc. of water at 37°. At definite time intervals, an estimate was made of the portion of the tablet remaining undissolved.

In Vivo.—At definite time intervals an estimate was made of the portion of the tablet remaining undissolved in the mouth of the mouse. The time necessary for each tablet to half dissolve *in vitro* and *in vivo* along with the melting point ranges (5) is given in Table II.

Phenobarbital.—As another means of comparing the rates of absorption from sublingual bases, sodium phenobarbital was incorporated into tablets. These tablets were administered to rabbits and periodic blood level determinations were made with the ultraviolet spectrophotometer. Blood samples were extracted with chloroform which were in turn extracted with 0.2 *N* NaOH. The ultraviolet absorption spectra of these solutions was studied at 255 mμ. The sensitivity of this method was of the order of magnitude of 0.5 mg./100 cc. of blood with less than 10% error (18, 19). Goldbaum's method (19), which does not differentiate between the barbiturate and its metabolic product (20), was used in this study.

Sodium phenobarbital was chosen because it is a long acting barbiturate and is slowly excreted over a period of several days; therefore, only negli-

[4] El Tronics, Counting Rate Meter, RM 481, Philadelphia, Pa.
[5] El Tronics, Scaler, Model S-64-S, Philadelphia, Pa.
[6] Esterline-Angus Graphic Ammeter, Model AW, Indianapolis, Ind.

TABLE I.—AVERAGE RATES OF SUBLINGUAL ABSORPTION OF RADIOACTIVE SODIUM IODIDE-131 FROM VARIOUS TABLET BASES

Tablet Bases, 1 μc. per Tablet	Counts per Sec., Absorption Time: 1 Hr. M[a]	1 Hr. S. E.[b]	2 Hr. M	2 Hr. S. E.	3 Hr. M	3 Hr. S. E.	4 Hr. M	4 Hr. S. E.	5 Hr. M	5 Hr. S. E.	3-Hr. Index
1. Lactose	0.8	0.2	1.7	0.3	2.6	0.4	3.5	0.3	3.8	0.2	53
2. Sucrose	0.7	0.2	1.6	0.2	2.6	0.4	3.5	0.3	4.7	...	53
3. Carbowax® 1540[c]	0.2	0.1	0.6	0.1	0.9	0.1	1.4	0.1	1.5	0.1	18
4. Carbowax® 4000[c]	0.6	0.1	1.2	0.1	1.4	0.1	1.7	0.1	2.0	0.2	29
5. Carbowax® 1000 MS[d, e]	0.1	0.1	0.5	0.1	1.0	0.1	1.4	0.1	1.6	0.1	20
6. Carbowax® 4000 MS[d, e]	0.1	0.1	0.5	0.1	1.0	0.2	1.6	0.3	1.8	0.3	20
7. Span® 65[f]	0	...	0	...	0	...	0	...	0	...	0
8. Tween® 61[f]	0.1	0	0.7	0.1	2.6	0.2	3.7	0.3	...	...	53
9. Myrj® 52[f]	0	...	0	...	1.1	0.1	2.3	0.2	2.8	0.1	22
10. Cocoa butter	0	...	...	...	1.7	0.2	1.9	0.2	2.2	0.2	35
11. Glyceryl MS[d, e]	1.0	0.2	3.3	0.2	4.8	0.3					98
12. Glyceryl MS-SE[d, e, g]	0.9	0.1	2.9	0.1	4.9	0.2					100
12a. Glyceryl MS-SE: 2.5 μc.	2.6	0.2	...	...	...	...					
12b. Glyceryl MS-SE: 5.0 μc.	4.8	0.5	...	...	...	...					
Aqueous solution	1.0	0.3	2.1	0.2	4.0	0.4					
Oral aqueous solution	5.1	0.1									

[a] Mean.
[b] Standard error of the mean.
[c] Carbide and Carbon Chemicals Co., New York, N. Y.
[d] Glyco Products Co., Brooklyn, N. Y.
[e] Monostearate.
[f] Atlas Powder Co., Wilmington, Del.
[g] Self-emulsifying.

gible quantities would be lost during the relatively short period in which the rate of absorption was studied.

Preparation of Tablets.—The tablet bases used in this study were the same as those previously compared by means of radioactive sodium iodide (Table I). Glyceryl monostearate, self-emulsifying, tablets were prepared with phenobarbital as well as the sodium phenobarbital. An aqueous solution of sodium phenobarbital as well as an alcoholic solution of phenobarbital was also prepared for sublingual administration.

The 3-cc. plastic planchets of 1-inch diameter which are commonly used in radioactivity studies were employed as a mold for those tablets made by fusion. Each tablet was made to contain 750 mg. of sodium phenobarbital. The sucrose and lactose base tablets were prepared according to the pharmaceutical method for preparing troches (21).

Rates of Disintegration.—These 3-Gm. tablets took much longer to disintegrate *in vivo* and *in vitro* than the 0.016-Gm. tablets of radioactive sodium iodide-131 which were prepared from similar bases. Nevertheless, the order of the rates of disintegration remained essentially the same as for the sodium iodide-131 tablets.

Administration of Tablets.—Young female rabbits weighing 1.5 Kg. ± 0.15 Kg. were anesthetized with an intraperitoneal injection of urethane, 1.5 Gm./Kg., and given 10 mg./Kg. of heparin, intravenously, as an anticoagulant. Neither urethane nor heparin exhibited any characteristic absorption peaks in the range that was used and any changes in their concentrations that might take place during the course of an experiment could have no significant effect on the determination of barbiturate levels. Fifteen minutes after the esophagus was ligated and the trachea cannulated, a blood sample was drawn off from the cannulated femoral artery and 5 cc. of this sample was analyzed as the blank. The tablet containing 750 mg. of sodium phenobarbital equivalent to a dose of 500 mg./Kg. was now administered sublingually. Two half-hour samples were taken and were followed by hourly samples. Three rabbits were used for each tablet base.

Blood Level Determinations.—The optical densities of the blood samples were determined and the initial blanks subtracted from these values as suggested by Goldbaum (19). The mean of the corrected optical density of each of the three samples was calculated in μg. per cc. of blood by comparison with the previously determined values for the standard dilutions. These average hourly values and the standard errors of the means are presented in Table III. In order more readily to evaluate the results, the μg./cc. levels after two hours were used to develop an arbitrary index for comparison, with the highest base equated to 100, and the other bases scaled accordingly.

TABLE II.—A COMPARISON OF IN VITRO AND IN VIVO RATES OF DISINTEGRATION OF SUBLINGUAL RADIOACTIVE SODIUM IODIDE-131 TABLETS

Bases Tested	Melting Point Range (5), ° C.	Time in Min. for Half Disintegration Of Tablets: *In Vitro*	*In Vivo*
1. Lactose	...	7	120
2. Sucrose	...	2	43
3. Carbowax® 1540	40–45	6	35
4. Carbowax® 4000	47–50	6	50
5. Carbowax® 1000 MS	38–40	8	...
6. Carbowax® 4000 MS	47–49	11	120
7. Span® 65	43–45	..	...
8. Tween® 61	34–38	90	10
9. Myrj® 52	41–44	11	10
10. Cocoa butter	30–32	7	10
11. Glyceryl MS	55–57	..	...
12. Glyceryl MS-SE	55–57	Swells	45

TABLE III.—AVERAGE RATES OF SUBLINGUAL ABSORPTION OF 500 MG./KG. OF SODIUM PHENOBARBITAL FROM VARIOUS TABLET BASES

Tablet Bases	μg./cc. Blood, Absorption Time										2 Hr. Index
	½ Hr.		1 Hr.		2 Hr.		3 Hr.		4 Hr.		
	M.[a]	S.E.[b]	M.	S.E.	M.	S.E.	M.	S.E.	M.	S.E.	
1. Lactose	4.5[c]	0.9	25.5	2.8	49.4	2.7	...	...	62.3	1.6	95
2. Sucrose	3.5	0.2	6.6	0.2	22.0	1.6	35.1	1.1	35.5	...	42
3. Carbowax® 1540	13.7	1.3	39.1	4.1	51.4	1.0	...	...	...	...	99
4. Carbowax® 4000	2.1	1.0	13.4	2.4	36.5	4.3	54.1	8.7	...	...	68
5. Carbowax® 1000 MS	3.1	0.2	26.7	0.5	52.2	1.3	...	...	...	...	100
6. Carbowax® 4000 MS	0.3	0.1	0.5	0.1	1.0	0.3	2.6	0.2	5.8	...	2
7. Span® 65	0.5	0.3	0.5	0.2	3.4	0.6	4.3	0.8	5.0	0.7	7
8. Tween® 61	2.2	0.8	12.3	2.7	27.1	3.3	49.1	6.3	62.5	1.6	52
9. Myrj® 52	2.5	1.2	5.1	1.7	23.5	2.1	62.4	8.3	...	...	45
10. Cocoa butter	0	0	3.8	0.8	6.0	1.2	8.9	0	11.8	0	12
11. Glyceryl MS	...	...	8.6	1.4	14.0	0.4	15.7	0.5	19.6	0.3	27
12. Glyceryl MS-SE	5.7	0.7	8.2	1.3	14.2	0.3	19.6	0.8	24.3	1.1	27
12a. Glyceryl MS-SE[d]	0.2	0	0.2	0	0.2	0	...	...	0.4	0.3	0
13. Glycerinated gelatin	2.8	0.4	18.1	1.1	29.8	1.3	27.4	0	...	...	57
Aqueous solution	20.5[c]	1.6	49.5	3.0	...	...	...	...			
Alcoholic solution[d]	10.0[c]	0.1	15.0	0.4	17.5	0.4	22.9	0.2			
Oral aqueous solution	54.4	0.7									

[a] Mean.
[b] Standard error of the mean.
[c] 20 minutes.
[d] Phenobarbital.

RESULTS

In the studies of the absorption of sodium iodide and sodium phenobarbital no relationship could be observed between the rates of absorption and either the *in vivo* or *in vitro* rates of disintegration. The *in vivo* and *in vitro* rates were not related. Comparisons may be best made by using the arbitrary indices for each study. No relationship can be observed between the relative rates of absorption of sodium iodide and sodium phenobarbital from the different tablet bases. Although the glyceryl monostearate bases have the highest rating of 98 and 100 for the liberation of sodium iodide, they only achieve a 27 rating for sodium phenobarbital. On the other hand, Carbowax® 1000 Monostearate and Carbowax® 1540 have a rating of 100 and 99, respectively, for sodium phenobarbital but only 20 and 18 for sodium iodide. Lactose and sucrose are both the same for sodium iodide at 53, but for sodium phenobarbital, lactose is rated at 95 while sucrose drops to 42. The only two bases which appear to be the same for both sodium iodide and sodium phenobarbital are Tween® 61 which is scaled at 53 and 52 and Span® 65 which is scaled at 0 and 7.

DISCUSSION

A method has been presented for utilizing radioactive isotopes in the comparison of rates of absorption from such forms of administration as sublingual tablets. It is expected that it will be equally advantageous in the study of other pharmaceutical dosage forms such as oral tablets, suppositories, and ointments. Determining the level of radioactivity through the tail is a much simpler and more convenient method than sacrificing animals and removing organs at periodic intervals.

However the use of radioactive sodium iodide-131 for the comparison of bases which are to be used for other active ingredients is not to be considered a valid procedure. The validity of the described method will depend on the use of the actual active ingredient, labeled with a radioactive element. Unfortunately, very few medicinals are at this time available with labeled atoms that have the proper emission characteristics. As more of these become available or as more sensitive methods of detection are perfected, the method will have greater applicability.

The dangers inherent in attempting to transfer information on behavior from one set of conditions to another have been well illustrated in this study. The rates of disintegration of the different tablet bases not only *in vitro* but also *in vivo* were not indicative of the rate at which sodium iodide would be absorbed from each tablet *in vivo*. Within a particular group of bases some relationship may be drawn but it is difficult to compare one type of base with another.

Sodium phenobarbital was incorporated into the same tablet bases as the radioactive sodium iodide-131 and administered sublingually to anesthetized rabbits. Periodic blood level determinations were made with the ultraviolet spectrophotometer. There was no correlation between the rates of absorption of sodium phenobarbital and radioactive sodium iodide-131. The rates of tablet disintegration could not be related to the rates of absorption.

These findings point to the same conclusions reached in many recent studies of absorption from ointments and suppositories. *In vitro* rates of disintegration or drug liberation do not necessarily compare with the *in vivo* behavior of a dosage form. A comparison of the rates of absorption of one drug from several bases should not be used in determining the base to be used with another drug. The factors influencing the rates of absorption for a specific drug should be determined and the proper vehicle selected by means of *in vivo* tests.

SUMMARY

1. Sublingual tablets with various bases were administered to anesthetized animals whose esophagus was ligated and trachea cannulated.

2. The rate of absorption of radioactive sodium iodide-131 from the tablets was traced

by measuring the level of radioactivity in the circulation through the tail of a mouse.

3. A spectrophotometric method for determining barbiturate blood levels was used for comparing the rates of absorption of sodium phenobarbital from different sublingual tablet bases.

4. No relationship exists between the rate of disintegration of the sublingual tablet bases tested and the rate at which radioactive sodium iodide-131 or sodium phenobarbital are absorbed from them.

5. No relationship exists between the rates at which radioactive sodium iodide and sodium phenobarbital are absorbed from the sublingual tablet bases tested.

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The Pharmacognosy of Smooth Brome Ergot*

By D. D. JONES and V. E. TYLER, Jr.

Ten samples of smooth brome ergot, collected in three midwestern states, were examined, described, and analyzed for moisture, fat, and alkaloid content. The moisture and fat concentrations were lower than those which have been reported for rye ergot, but the average alkaloid content of the samples slightly exceeded, and in the case of ergonovine more than doubled the N. F. IX requirement. Paper partition chromatography verified the identity of the water-soluble alkaloid as ergonovine and established the presence of both ergotamine and ergotoxine in the water-insoluble alkaloid group. Cultures prepared from these samples showed normal saprophytic growth and were capable of parasitizing rye.

CLAVICEPS PURPUREA (Fries) Tulasne has been reported to parasitize a number of North American grasses, including *Bromus inermis* Leyss, commonly referred to as smooth brome (1). This grass, in addition to being a common forage crop in the midwestern states, is frequently planted along the shoulders of highways, since its extensive root system is an efficient means of preventing soil erosion.

Observations made in the early summer of 1954 revealed that smooth brome growing along the highways in sections of Nebraska, Iowa, and Kansas was heavily ergotized, and collections of mature sclerotia were made in those states during June and July. Although numerous infestations of smooth brome were noted in close proximity to fields of wheat, rye, and oats, it was observed that none of these grains was infected by the fungus. It appeared to be of interest to determine if this phenomenon was due to the difference in flowering time between the smooth brome and the other crops or if it might be caused by a strain specificity of the fungus.

Examination of the literature revealed little information regarding the pharmacognosy of ergot of *Bromus inermis*, and the following study was undertaken to determine the morphology and histology of the fungus, its fat and moisture content, the concentration and identity of its alkaloids, and some of the characteristics of its saprophytic and parasitic growth and development.

* Received April 4, 1955, from the Department of Pharmacognosy, University of Nebraska, College of Pharmacy, Lincoln.

EXPERIMENTAL

Collection and Description.—Ten samples of smooth brome ergot were collected in the following locations on the dates indicated.

Sample No. 1 Lancaster County, Nebraska, site 1, June 27, 28
Sample No. 2 Lancaster County, Nebraska, site 2, June 30
Sample No. 3 Lancaster County, Nebraska, site 3, June 30

Sample No. 4 Fremont County, Iowa, site 1, July 4
Sample No. 5 Fremont County, Iowa, site 2, July 4
Sample No. 6 Stanton County, Nebraska, July 4
Sample No. 7 Otoe County, Nebraska, July 5
Sample No. 8 Merrick County, Nebraska, site 1, July 10
Sample No. 9 Merrick County, Nebraska, site 2, July 10
Sample No. 10 Washington County, Kansas, July 16

Samples of the sclerotia were examined with the aid of a hand lens, and cross and longitudinal sections were cut by supporting the untreated sclerotia in paraffin and cutting on a Spencer rotary microtome to a thickness of 8 μ. The consistency of the sclerotia was such that sections prepared in this manner showed a minimum of distortion and were far superior to sections prepared in a similar fashion following softening of the sclerotia by soaking in 50% hydrofluoric acid for various periods of time. Mounted in chloral hydrate solution, the sections proved quite suitable for microscopic examination and this mounting medium served to emphasize the pigmentation in the outer cells.

The smooth brome ergot sclerotia were generally cylindrical or slightly angular, frequently curved and tapering to more or less pointed ends. They ranged in length from 5 to > 19 mm. and in diameter up to 2 mm. The surface was furrowed longitudinally and occasionally also transversely, giving it a somewhat wrinkled appearance. Color varied from purple brown to nearly black. The fracture was short, and the internal color varied from white to pink or violet. Odor and taste were characteristic. Typical examples of the sclerotia are shown in Fig. 1.

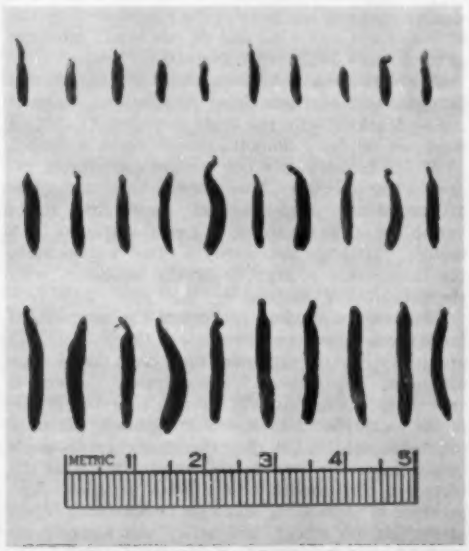

Fig. 1.—Typical sclerotia of smooth brome ergot.

Cross sections showed an outer layer about 15 to 25 μ in thickness, composed of compact pigmented hyphal cells. Internally, a large area of pseudoparenchyma was formed by colorless hyphal cells ranging from 3 to 15 μ in diameter with the majority ranging between 7 and 11 μ. In longitudinal section, many of these hyphal cells appeared elongated and lengths up to 26 μ were observed.

Moisture Determination.—Part of each collection was ground to a No. 20 powder in a Wiley laboratory mill and two 500-mg. portions of each sample were accurately weighed on an analytical balance, placed in a tared porcelain evaporating dish and heated at 105° in an oven until constant weight was attained. This usually required a minimum period of eight hours. The average moisture content (loss of weight on drying) was then calculated with the results indicated in Table I.

TABLE I.—RESULTS OF ANALYSES OF SMOOTH BROME ERGOT

Sample No.	Moisture, %	Fat, %	Water-soluble[a] Alkaloids, %	Water-insoluble[b] Alkaloids, %
1	8.1	10.6	0.019	0.079
2	7.1	8.3	0.018	0.107
3	7.7	9.4	0.025	0.129
4	3.4	12.1	0.030	0.180
5	3.9	10.8	0.024	0.165
6	2.9	13.5	0.037	0.245
7	6.5	12.6	0.027	0.178
8	4.7	16.4	0.037	0.248
9	4.7	14.4	0.026	0.213
10	2.8	26.9	0.012	0.161
Average	5.2	13.5	0.026	0.171

[a] Calculated as ergonovine, on basis of moisture-free and fat-free samples.
[b] Calculated as ergotoxine, on basis of moisture-free and fat-free samples.

Fat Determination.—The fat content was determined using duplicate 500-mg. portions of each powdered sample, accurately weighed. Extraction was carried out with petroleum ether in Soxhlet extractors for twenty hours. The average fat content of each sample was calculated and is reported in Table I.

Alkaloidal Assay.—Portions of the defatted ergot samples obtained as described above were milled to a No. 60 powder and duplicate 100-mg. quantities of each sample were assayed for water-soluble and water-insoluble alkaloids according to the method of Silber and Schulze (2).

By this method the total alkaloids are extracted from the drug by shaking with ammoniacal ether and the water-soluble alkaloid selectively removed from the ether extract by shaking with *p*H 8 phosphate buffer. The water-insoluble alkaloids are then obtained from the ether extract by shaking with 2% tartaric acid solution. Each alkaloid group is then determined quantitatively by mixing with a *p*-dimethylaminobenzaldehyde reagent, illuminating with a mercury vapor light and estimating the color formed by photoelectric colorimetry.

Minor modifications were introduced for this experiment and consisted of illuminating the mixture of reagent and alkaloid solution with a mercury vapor lamp, designed for use with the Fisher Nefluoro-Photometer, at a distance of 24 cm. for the specified period of time. This solution was then transferred to a 1-cm. cuvette and the absorbance

determined in a Beckman, model D.U., spectrophotometer equipped with photomultiplier attachment, at 590 mμ.

Alkaloid concentrations were determined from a standard curve prepared with ergonovine maleate, U. S. P. Reference Standard, in concentrations ranging from 5 to 80 μg. per ml. Concentrations of water-soluble alkaloid were calculated as ergonovine and water-insoluble alkaloids as ergotoxine. Average figures for the duplicate analyses are expressed on a percentage basis of the weight of moisture-free and fat-free ergot samples in Table I.

Identification of the Alkaloids.—Verification of the identity of the water-soluble alkaloid as ergonovine and the determination of the composition of the water-insoluble group were undertaken using paper partition chromatography.

Duplicate quantities of the buffered extracts of Samples No. 1–10 containing the water-soluble alkaloid were spotted in 40 μl. amounts on $18^1/_4$ x $22^1/_2$ in. sheets of Whatman No. 1 filter paper and chromatographed with a wash liquid consisting of the upper phase of a mixture of butanol-acetic acid-water (4:1:5) as described by Tyler and Schwarting (3). Control spots on the same sheets consisted of 20 μl. of a standard ergonovine maleate solution (200 μg./ml.) to which was added 20 μl. of the *p*H 8 phosphate buffer solution used for shaking out the water-soluble alkaloid. Under these conditions the ergonovine standard showed an average R_f value of 0.73 and each of the 10 extracts showed a single blue fluorescent spot which possessed an R_f varying from 0.72 to 0.75 and averaging 0.73. From this data it was concluded that the water-soluble alkaloid present in each sample was ergonovine.

Separation and identification of the water-insoluble alkaloids were next undertaken. The tartaric acid shakeouts of Samples No. 1–10 were made alkaline to litmus with diluted ammonium hydroxide and shaken out with 1 ml. of ether for three minutes. The ether extracts thus obtained were spotted in duplicate in 20 μl. portions on 1 x $22^1/_2$ in. strips of Whatman No. 1 filter paper, previously dipped in McIlvaine's buffer at *p*H 4 and air dried. Descending formation was carried out with a wash liquid composed of chloroform saturated with 0.1 *M* aqueous citric acid solution as described by Brindle, *et al.* (4).

Two distinct fluorescent spots were observed on each of the test strips. The first spot varied from R_f 0.14 to 0.20 with an average of 0.18. The second showed R_f values between 0.54 and 0.66 with an average of 0.60. Although the average figures were in good agreement with those reported by Brindle, *et al.* (4) for ergotamine and ergotoxine, respectively, it was felt that the cause of the undue fluctuation in R_f values should be determined.

Experiments indicated that the variation was due to quantitative differences in the amount of alkaloid spotted. For example, with ergotamine R_f values between 0.12 and 0.34 were obtained, the R_f increasing with the concentration. Variations between 0.54 and 0.72 were obtained in like manner for ergotoxine. However, average R_f values for ergotamine and ergotoxine were 0.20 and 0.62, respectively, which coupled with the fact that the unknowns showed variations well within the range of variation of the controls, substantiated the conclusion that the water-insoluble alkaloid group of each sample consisted of both ergotamine and ergotoxine.

Culture Studies.—Saprophytic cultures of several of the smooth brome ergot samples were prepared by cutting small segments from the inner pseudoparenchyma tissue and sterilizing by dipping in diluted Lugol's solution followed by rinses in alcohol, sterile distilled water, and rapid passage through the flame of a Bunsen burner. These bits of sclerotia were implanted on Sabouraud's dextrose agar (5) and began to develop the typical mycelial growth of *Claviceps purpurea* in a very few days. Transfers from such cultures, introduced into a mannitol-casein hydrolysate medium which was subjected to constant shaking, showed the typical growth form described by Tyler and Schwarting (6).

Aqueous suspensions of the growth on solid medium as well as portions of the liquid medium containing mycelium and spores were utilized to determine if the smooth brome ergot strains could parasitize another host; in this case, rye. The inoculum was applied in the usual fashion to rye flowers which had reached the proper stage of development. Honey dew formation was observed within 7 to 10 days and sclerotia, typical in every respect of rye ergot, were formed.

DISCUSSION

The morphological and histological features of smooth brome ergot are quite similar to those of rye ergot except the sclerotia of the former are considerably smaller in size. This variation is to be expected since it is well recognized that the size and shape of ergot sclerotia are modified by the species of grass which is infested.

The moisture content, with the exception of the first sample collected, was considerably below the 8% maximum limit of the N. F. IX (7). This may be partially explained by the fact that the smaller sclerotia are more easily dehydrated by the action of sun and wind and do not retain moisture as tenaciously as do larger sclerotia.

One of the most surprising analytical results was in regard to the fat content of the samples. According to Markwell (8), rye ergot contains 30–40% of fixed oil or fat. Smooth brome ergot averaged 13.5% of fat and only one sample appreciably exceeded the average. This low fat content may be attributed to environmental conditions, strain variability of the organism, or a combination of both factors. It is not necessarily a disadvantage since the fat content of ergot is closely associated with the instability of the drug.

The average alkaloid content of the samples exceeds the minimum requirements of the N. F. IX (7) and in the case of ergonovine more than doubles the minimum requirement. Considerable differences were noted in the specific samples collected in different localities, as indicated in Table I. It is of interest to note that all three classes of ergot alkaloids (ergonovine, ergotamine, and ergotoxine) were detected in the smooth brome ergot samples. The presence of these three alkaloids in American ergots of rye, durum wheat, and barley was recently reported for the first time by Svoboda, *et al.* (9).

The smooth brome strains grew normally in saprophytic culture and were capable of parasitizing rye. This is in agreement with the work of McFarland (10) who investigated the parasitic ability of a smooth brome ergot and found it to be capable of

infecting rye. It must be concluded, therefore, that the lack of ergot infection in grain fields adjacent to infected smooth brome is due primarily to the difference in flowering time of the plants and not to strain specificity of the fungus.

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The Effect of Topically Applied Hyaluronidase on Cutaneous Permeability to Certain Substances*

By HAROLD SHEINAUS†, JOHN E. CHRISTIAN, and GLEN J. SPERANDIO

Studies were made to determine if hyaluronidase, when incorporated into ointments, affected the penetration of medication contained in ointments applied to the skin. Four substances were tested: sulfanilamide, sodium sulfanilamide, sodium iodide, and sodium chloride. Three different ointment bases were used: an oleaginous base, an absorption base, and a washable emulsion type base. The ointments were applied to the intact skin of guinea pigs, and a technique using radioactive isotopes is reported for determining the extent of penetration of the labeled medicaments. It was found that the substances studied showed a limited degree of penetration into the skin from the bases used, but except in the case of sodium iodide the penetration was not appreciably affected by hyaluronidase.

INVESTIGATIONS of percutaneous absorption, penetration, and permeability have been carried out extensively by many workers. However, little has been done in regard to investigating the effect of agents added to ointments to increase penetration of the medicament contained in them.

Hyaluronidase is currently attracting widespread attention in the biochemical and medical fields. Numerous clinical uses for this enzyme already have been established, and many more are now in the investigative stages. Because of the presence of its substrate in dermal connective tissue, it was thought it might affect the penetration of drugs applied topically.

Hyaluronidases are the enzymic substances which either hydrolyze or depolymerize the substrate in dermal tissue. This results in a disappearance of the characteristic reactions of the intact polymer, the reduction in viscosity being the key to permeability changes. The enzyme is rapidly destroyed by heating to 65°. In a dry state, it is stable at room temperature for more than one year (1) and if stored in the refrigerator at below 50° F., it is stable indefinitely (2). In aqueous solution it is stable up to seven days at room temperature and for three to four weeks at refrigerator temperature (2).

Hyaluronidase was found by Kirby, *et al.* (3), to increase the area of anesthesia by procaine, when both were administered together subcutaneously. Asboe-Hansen (4) reported on the action of injected hyaluronidase on the permeability of human skin, in regard to spreading of allergen and scratch test reactions. Popkin (5) found that hyaluronidase could be effectively transferred through the unbroken skin by iontophoresis, and was successful in treating scleroderma and skin ulcers by this method.

Bresgen (6) applied an insulin-hyaluronidase powder mixture topically to rabbit skin, and reported an effect on the blood sugar which was not demonstrable if hyaluronidase were absent.

Parvis and Marazzi (7) applied penicillin and hyaluronidase in an anhydrous ointment base to guinea pig skin, reporting increased horizontal spreading of the penicillin when hyaluronidase was present.

The specific objective of this study was to determine the effect of hyaluronidase on the cutaneous penetration of certain substances applied topically in ointment bases.

* Received February 24, 1955, from the School of Pharmacy, Purdue University, Lafayette, Ind.
Presented to the Scientific Section, A. PH. A., Miami Beach meeting, May, 1955.
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The substances, the penetrations of which were studied, were sulfanilamide, sodium sulfanilamide, a soluble cation (Na^+), and a soluble anion (I^-). The bases used were Plastibase,[1] (an anhydrous base composed of five per cent polyethylene resin in liquid petrolatum), Plastibase hydrophilic (an absorption base), and hydrophilic ointment U. S. P. XIV.

EXPERIMENTAL

General Plan.—With the specified objective in mind, ointments containing radioactive tracers and hyaluronidase were applied to the skin of guinea pigs. The animals were sacrificed, and skin punches were made and sectioned parallel to the skin surface on a freezing microtome. Each section was processed according to a valid method of sample preparation, depending on the isotope used. These samples were counted for radioactivity, compared with controls, and the degree of penetration of the drugs in the various bases was determined.

The radioactively tagged sulfanilamide was synthesized from acetanilid and S^{35}-labeled sulfuric acid according to the method of Byrne, Alberts, and Christian (8). A 23.5 per cent yield was obtained having a specific activity of approximately 0.6 mc./Gm.

Chemical purity was further checked by chromatographing the synthesized product and U. S. P. XIV sulfanilamide simultaneously, and radiochemical purity was established in order to obviate the possibility of following an impurity containing the radioactive label.

For use in testing the penetration of a soluble sulfonamide, S^{35}-labeled sodium sulfanilamide was prepared by the interaction of labeled sulfanilamide with an equimolar quantity of sodium hydroxide, and subsequent recrystallization. An 80% yield was obtained.

The following ointments were utilized in this study: S^{35}-labeled sulfanilamide in Plastibase, S^{35}-labeled sulfanilamide in Plastibase hydrophilic, S^{35}-labeled sodium sulfanilamide in Plastibase hydrophilic, S^{35}-labeled sodium sulfanilamide in hydrophilic ointment U. S. P. XIV, sodium radio-iodide (I^{131}) in Plastibase, sodium radio-iodide (I^{131}) in Plastibase hydrophilic, sodium radio-iodide (I^{131}) in hydrophilic ointment U. S. P. XIV, radio-sodium (Na^{22}) chloride in Plastibase, radio-sodium (Na^{22}) chloride in Plastibase hydrophilic, radio-sodium (Na^{22}) chloride in hydrophilic ointment U. S. P. XIV.

All ointments were prepared by thorough levigation on an ointment slab. The sulfanilamide and sodium sulfanilamide were incorporated in a strength of 5%, which gave the ointments an activity of approximately 25 mc./Gm. at the time of use. The ointments containing I^{131} and Na^{22} were prepared to contain an equal amount of activity per Gm.

To a portion of each, at the time of use, was added 750 TRU[2] of sterile lyophilized hyaluronidase[3] per gram of ointment. These are designated as the test ointments.

Ointments prepared with Plastibase were anhydrous. Ointments prepared with Plastibase hydrophilic were w/o emulsions, with tracer and enzyme both in solution in the internal phase. Ointments prepared with hydrophilic ointment U. S. P. XIV were o/w emulsions with tracer and enzyme both in solution in the external phase.

Ointments were freshly prepared on the day of use and kept under refrigeration until used. The hyaluronidase stocks from which the test ointments were prepared were also kept under refrigeration.

Calculations of the radiation dosages received by the enzyme, in combination with the experiences of Schoenberg, *et al.* (9) on the effect of radiation on hyaluronidase, indicate that the factor of radiation damage to the enzyme is negligible.

Procedure Used.—The guinea pig was sedated with sodium phenobarbital, 100 mg./Kg. intraperitoneally, which was sufficient to maintain sedation for the duration of the experiment. One hour after injection, the hair of the back was clipped with an Oster electric small animal clipper, and a 7 x 7 cm. area in the center of the back was closely shaved with a Sunbeam electric razor. The shaved area was carefully examined with the aid of a hand lens to insure that no irritation or other abnormal appearance was present. Four circles of 17-mm. diameter, two on each side of the spine, were marked out using the open end of a glass vial and an inked stamp pad. The two areas on the right side were used as control sites and two areas on the left side were used as corresponding test sites.

A Pyrex glass cylinder of 14-mm. external and 11-mm. internal diameter, and 10 mm. high, was

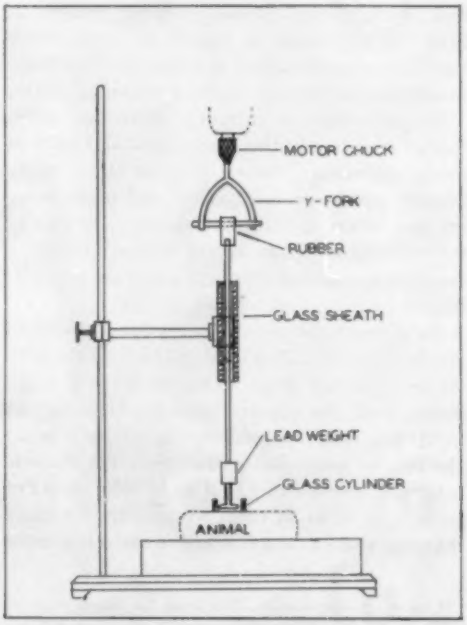

Fig. 1.—Diagrammatic sketch of mechanical rubbing device.

[1] Plastibase is a trade mark of E. R. Squibb and Sons.
[2] Turbidity reducing units.
[3] Enzodase, E. R. Squibb and Sons, New York; and Hyasyme, Abbott Laboratories, North Chicago, Ill.

cemented with Duco household cement to the skin in the center of each circle, and allowed to dry for one hour. The animal was placed on a board and its legs taped down.

To achieve uniformity of rubbing, the mechanical device illustrated in Fig. 1 was used.

The glass rod assembly, consisting of a glass rod flattened to a disk at one end, the lead weight, the rubber cap, and the crossbar, had freedom of movement in a vertical direction. This maintained a constant rubbing pressure on the animal, regardless of its breathing or other slight movements. The weight of the glass rod assembly was 43.7 Gm.

Exactly 0.1 Gm. of ointment was added to the bottom of the disk of the tared glass rod assembly. The latter was inserted into its sheath and the rubber cap with crossbar put on. The assembly was positioned over the appropriate cylinder, and lowered carefully until the full weight of the assembly was being supported by the animal. The motor and Y-fork were lowered to straddle the crossbar, and rotational rubbing was carried out at 48 r. p. m. for fifteen minutes.

At the end of this time, the device was removed and the animal allowed to remain undisturbed for four hours. The cylinders were then removed, and excess ointment removed carefully by cotton-tipped swabs moistened with alcohol (for oleaginous bases) or water (for hydrophilic ointment). The animal was then killed by a sharp blow on the head, and the skin excised, cutting well outside the treated areas. The skin was placed on a sheet of saline-moistened parchment paper, and these both were tacked to an asbestos board without distortion to the skin.

Circular skin punches were made with a 17-mm. brass cork borer on the previously delineated circles. Each skin punch was placed on the grooved stage of a Bausch and Lomb clinical microtome with freezing attachment, epidermis up. After leveling the surface with a clean glass microscope slide, it was frozen and sectioned parallel to the surface at 50 μ. In order to obtain maximum recovery of sections, a five-second cutting cycle was found to be best.

Each section, as it was cut, was removed from the knife edge with an individual glass rod. Rod and section were placed in a serially numbered 2-inch test tube into which had previously been pipetted 0.2 ml. of nitric acid (when S^{35} or Na^{22} was used) or 0.2 ml. of 15% sodium hydroxide (for I^{131} samples). After the first five sections were cut, the microtome knife was shifted laterally in its holder about 2 cm. in order to present a fresh portion of the knife edge uncontaminated by residual ointment from the surface. After cutting 47 sections, the residue, if any, was placed in a separate test tube.

The contents of the tubes were digested over a boiling water bath for fifteen to thirty minutes, depending on the digesting liquid, until complete solution occurred. Samples for counting were prepared by transferring the contents of each tube, when cool, into a cupped aluminum sample pan which in the cases of S^{35} and Na^{22} contained disks of lens paper and in the case of I^{131} were coated with paraffin and fitted with disks of filter paper.

Counting of all samples was done with an end-window G-M tube having a window thickness of 1.8 mg./cm.^{2}, and with maximum approach to 2π geometry. All activities were corrected for resolution, background, absorption, and decay as required.

TABLE I.—TOTALIZED RADIOACTIVITY IN ALL SECTIONS OF SKIN PUNCHES FROM SAMPLES OF SKIN TREATED WITH OINTMENTS CONTAINING RADIOACTIVE LABELED COMPOUNDS, WITH AND WITHOUT HYALURONIDASE

	Sample	Activity (c. p. m.)[a] Control[b]	Test[b]
S^{35}-labeled sulfanilamide in Plastibase	1	834	500
	2	369	1,183
	3	526	47
in Plastibase hydrophilic	1	593	614
S^{35}-labeled sodium sulfanilamide in Plastibase hydrophilic	1	66	143
	2	152	88
	3[c]	149	173
in hydrophilic ointment U. S. P. XIV	1[c]	221	145
Sodium radio-iodide (I^{131}) in Plastibase	1	781	4,542
	2	706	7,975
	3	1,099	5,413
	4	118	576
	5	3,787	29,279
	6	2,174	3,800
in Plastibase hydrophilic	1	2,576	2,719
	2	1,545	2,018
	3	2,492	1,833
	4	4,774	872
in hydrophilic ointment U. S. P. XIV	1	456	157
	2	60	204
	3	21,652	18,693
Radio-sodium (Na^{22}) chloride in Plastibase	1	97	399
	2	168	501
	3	1,724	582
	4	355	209
	5	2,051	655
	6	198	1,963
in Plastibase hydrophilic	1	3,168	2,461
	2	3,436	6,000
in hydrophilic ointment U. S. P. XIV	1	3,457	4,331
	2	1,843	3,305

[a] Each figure represents the sum of the corrected activities expressed in counts per minute of the 48 individual sections of a skin punch, exclusive of the first five sections.
[b] All test ointments and control ointments were identical, except for inclusion of 750 TRU hyaluronidase per gram of ointment in the former.
[c] These samples were run on a 3,600-Gm. male rabbit.

RESULTS AND DISCUSSION

A summary of the results of sample counting appears in Table I. It is valid to make a quantitative comparison only between test and control counts in a given sample. Other comparisons can be general only, and must take account of the differing conditions under which the figures for comparison were obtained.

Two measurable attributes of the penetration process were used as criteria of penetration enhancement in this study. One was the totalized count in all the sections of a given skin punch exclusive of the first five sections. The other was the distribution of the counts within the punch.

It was judged that a positive effect by the enzyme should be characterized both by a higher total count, and by a count distribution such that a large majority of individual counts from test site sections be greater than the individual counts from corresponding control site sections. This superiority was also required to hold when the control total was increased by counts of its fourth and fifth sections, and the test total reduced by the counts of its sixth and seventh sections. The possible error in matching corresponding sections has been estimated as plus or minus two sections. From Table I, it can be seen that a limited degree of penetration was obtained with all substances and all bases tested. In all cases but one, hyaluronidase was observed to have no appreciable effect on penetration.

From the data in this Table, and from distribution studies made within each sample, it can be reported that hyaluronidase had a positive effect on the penetration of sodium radio-iodide (I^{131}) from Plastibase into guinea pig skin. This is based on the results of six samples, run on four different animals. Similar effects by hyaluronidase were not obtained with any other combination of tracer and base.

An example of the radioactivity of the individual sections of one skin punch is plotted in Fig. 2. This is a plot of the radioactive counts obtained from consecutive parallel sections, starting with the outermost, of a skin punch treated with sodium radio-iodide (I^{131}) and hyaluronidase in Plastibase (Sample 1, Test, of Table I). The positive effect obtained with hyaluronidase can be seen by the distribution comparisons shown in this figure. The peaks on this graph presumably represent the levels at which a comparatively large number of hair follicles are rooted.

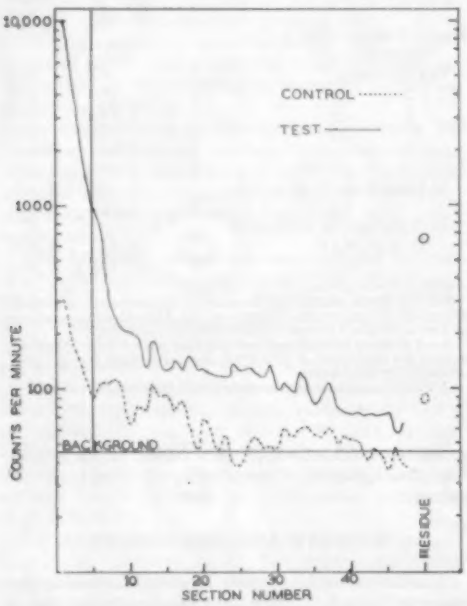

Fig. 2.—A semilog plot of radioactive counts *vs.* section number. Sample 1, sodium radio-iodide (I^{131}) in Plastibase.

SUMMARY AND CONCLUSIONS

An attempt has been made to determine the effect of topically applied hyaluronidase on cutaneous permeability to several radioactive-labeled substances contained in three different ointment bases.

Methods of sample preparation have been developed for use in determination of S^{35}, I^{131}, and Na^{22} activity in skin tissues, and are reported.

Techniques and procedures for measuring permeability of animal skin to topically applied agents are reported.

It was found that the substances studied showed a limited degree of penetration into the skin from the bases used. In the majority of cases, penetration and/or permeability was not appreciably influenced by topically applied hyaluronidase.

Hyaluronidase was found to enhance considerably the penetration of sodium radio-iodide (I^{131}) from Plastibase into guinea pig skin when both were applied together by inunction.

As a result of this work, it can be concluded that topically applied hyaluronidase is able to increase cutaneous permeability to certain topical medication under certain conditions. It is suggested that, although it has shown a high degree of selectivity in producing an effect in these experiments, hyaluronidase may be found useful for specific penetrants not investigated in this work.

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A Study of Emulsification with Ultrasonic Waves I*

By HAROLD M. BEAL† and DONALD M. SKAUEN

An attempt has been made to study and evaluate some of the factors involved in emulsification with ultrasonic energy. The stability of the emulsifying agent to ultrasonic waves is a factor in its choice in emulsification. Two exposure chambers were found to be superior; one a cellulose acetate chamber and the other a Pyrex test tube fitted with a brass diaphragm. These two types produced the best emulsions and showed no appreciable stationary wave pattern found to be unfavorable to emulsification. Orientation of the exposure chamber was shown to be important in emulsification with ultrasonic waves. A distance of an exact number of half wavelengths between the bottom of the exposure chamber and the crystal was shown to deliver maximum acoustical energy to the contents of the chamber and at these points emulsification was best.

THE RANGE OF SOUNDS audible to the human ear is considered to be from 20 to about 20,000 vibrations a second. Waves in the range above 20,000 vibrations a second (20 kc. a second) are referred to as "supersonic" or "ultrasonic," the term ultrasonic being most commonly used today.

Waves above the audible range were recognized early in the 19th century; however, they remained a laboratory curiosity until World War I, when they were used in a device for the detection of undersea craft.

Wood and Loomis (1) and Richards and Loomis (2) indicated in 1927 ultrasonic's potential in two reports; the first concerning the physical and biological effects of high-frequency sound waves and the second regarding their chemical effects. They reported some of the spectacular results of the application of ultrasonic energy. Since that date a great amount of interest has been evidenced by various investigators in the chemical and biological fields concerning its chemical and physical effects upon various substances. This interest has extended to the pharmaceutical field where the principle might be applied in the preparation of superior emulsions and suspensions. Several articles have been published in recent years regarding emulsification with ultrasonics; however, most reports are concerned with volatile substances such as xylene, toluene, etc. Campbell and Long (3) carried on some experiments with fixed oils. Audouin and Levavasseur (4) made a study of emulsification of vegetable oils with ultrasound, with particular emphasis on the effect of frequency.

* Received March 31, 1955, from the University of Connecticut School of Pharmacy, Storrs.
Presented to the Scientific Section, A. PH. A., Boston meeting, August, 1954.
The paper is based on a dissertation submitted by Harold M. Beal to the Graduate School of the University of Connecticut in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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The authors are indebted to Dr. Raymond H. Wallace, Associate Professor, University of Connecticut for his invaluable aid and advice in the use of ultrasonics.

The ultrasonic generator used in the present investigation was a General Electric Model G-3; supplying four frequencies—300, 500, 750, and 1000 kc., with a maximum oscillator plate power of 300 w. At full power the oscillator delivered approximately 95 w. acoustical energy to the sound chamber, as determined calorimetrically.

EXPERIMENTAL

An exposure chamber of the following construction was used in the investigation (Fig. 1): the bottom was removed from a Pyrex test tube of approximately 35.5 mm. inside diameter, 1.5 mm. wall thickness, and 20 cm. length. The cut surface was ground to smoothness. A brass threaded fitting was cemented to the walls of the tube and the edge ground to a smooth surface. A brass collar permitted the use of diaphragms of various materials and allowed for easy replacement. Diaphragms of aluminum, copper, cellulose acetate, and brass were used, with brass shimstock (0.001 in. thick) proving the most satisfactory.

For microscopic examination and comparison of the emulsions a "globule counting method," devised by Smith and Grinling (5) and expanded by Cockton and Wynn (6), and Levius and Drommond (7) was used. A Helber counting chamber and a hemacytometer cell were employed. The central por-

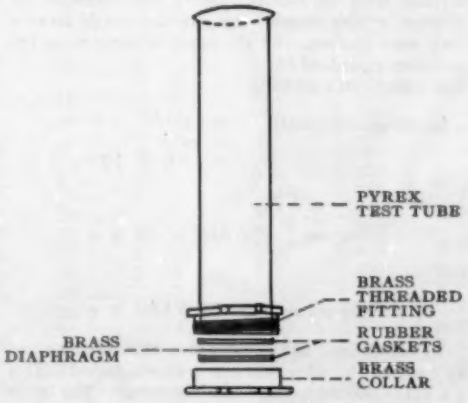

Fig. 1.—Exposure chamber.

tion of the hemacytometer cell is 0.1 mm. deep and that of the Helber cell 0.02 mm. deep. In both cells this central portion is ruled into squares 0.0025 mm.^{2} in area. The microscope used was equipped with a 43× high power and a 96× oil-immersion lens with a 10× eyepiece. With the 43× lens the counting chamber rulings were out of focus with the oil globules, making an accurate count difficult to perform. To overcome the difficulty a ruled ocular disk was placed in the eyepiece and the area of the squares on the disk determined. With the oil-immersion lens use of the ruled ocular disk was unnecessary.

A 50% aqueous propylene glycol solution was used as a diluting fluid to prevent Brownian movement of the oil globules. The emulsion was diluted so that when the cell was filled and covered with a cover glass a countable number of globules appeared in each square of the ruled disk or the counting chamber rulings. Dilution varied from 1 in 250 to 1 in 1,000.

When an even distribution was obtained the number of globules was counted in 20 small squares using the oil-immersion lens and 81 squares with the 43× lens. The cell was searched to its complete depth for globules that did not cream to the surface. To avoid counting the same globule twice the count for each square included all globules that lay on or touched the top and left-hand sides and excluded all those that lay on or touched the bottom and right-hand sides of the square. In each case duplicate dilutions and counts were taken.

From the total number of oil globules counted the following values were determined:

(*a*) H, which expresses the number of millions of globules into which 1 cu. mm. of the oil has been subdivided.

The volume under 20 small squares in the Helber chamber would equal:

$$20 \times {}^1/_{50} \times {}^1/_{10} \times {}^1/_{20} = {}^1/_{1,000} \text{ cu. mm.}$$

If N is the number of globules counted in this volume of a 1 in Z dilution of an emulsion containing 25% v/v of oil, then:

$$H = N \times 1{,}000 \times Z \times (100/25) \times 10^{-6} \text{ millions}$$

(*b*) D, the "root mean cube diameter," which is derived from H and represents the diameter (in microns or millimeters) the globules would have if they were uniform and the same in number as the emulsion examined (6).

The volume of a globule

$$\begin{aligned} \text{(assumed spherical)} &= 4\pi r^3/3 \\ &= \pi d^3/6 \\ &= 1/(H \times 10^6) \end{aligned}$$

Therefore

$$D \text{ (in mm.)} = \sqrt[3]{6/H \times 10^6 \times \pi}$$

and

$$D \text{ (in microns)} = 10\sqrt[3]{6/H \times \pi}$$

(*c*) S, defined as the total area of interface given by 1 ml. of the oil which has been emulsified. This is a value related to "specific interface." The latter expression is defined as the total area of interface given by 1 Gm. of the internal phase (7).

Using the value of D in cm., the surface of one globule $= \pi D^2$ sq. cm. and $S = \pi D^2 \times H \times 10^6 \times 10^3$ sq. cm./ml. of oil or $S = \pi D^2 \times H \times 10^9$ sq. cm./ml. of oil.

For this investigation calculation of the three values; H, D, and S was considered to give a satisfactory basis for comparison of the various emulsions.

Effect of Ultrasonic Waves upon Emulsifying Agents.—Intense ultrasonic waves are known to bring about chemical changes in compounds which come into contact with them. In solutions of the type represented by the gums, soaps, etc., there exists a type of structural arrangement possessing properties similar to those of an elastic solid. Such fluids are said to possess structural or anomalous viscosity. Freundlich and Gillings (8) have shown that ultrasonic waves destroy this structure and thereby reduce the viscosity of the solutions.

Solutions of the emulsifying agents used in the present investigation were treated with ultrasonic waves for periods of seven and a half, fifteen, and thirty minutes. Polyethylene glycol 400 monostearate, tragacanth, and hard soap solutions showed significant losses in viscosity while those of acacia, sodium lauryl sulfate, and polysorbate 80 showed no significant change (Table I).

Since emulsifying agents used in pharmaceutical emulsions contribute to stability in part due to the viscosity of their solutions a lowering of viscosity would be undesirable. In emulsification with ultrasonic energy this would necessarily be a consideration in the selection of an emulsifying agent.

Effect of Type of Exposure Chamber in Emulsification with Ultrasonic Waves.—There has been little information in the literature relative to exposure vessels for preparing emulsions with ultrasonic energy. A series of emulsions was made using five different exposure chambers to determine whether the type of container played a role in the quality of the emulsion produced.

A 25% light liquid petrolatum emulsion was made using 1% hard soap as the emulsifying agent. The emulsion was made in duplicate in each of the five exposure chambers. The base of each container was positioned exactly 33.5 mm. above the crystal face. Exposure time was ten minutes, frequency 300 kc., and plate current 200 ma. (approximately 95 w. acoustical energy). By means of a cooling coil in the sound chamber of the transducer assembly the bath was brought to 25° at the start of each exposure. The following exposure chambers were used:

Chamber I.—A cellulose acetate flat-bottomed tube, wall thickness 0.5 mm., inside diameter 42 mm., and height 19 cm.

Chamber II.—Exposure chamber shown in Fig. 1.

Chamber III.—Pyrex hemispherical bottomed test tube, wall thickness 1.5 mm., inside diameter 21.5 mm., and height 20 cm.

Chamber IV.—Pyrex hemispherical bottomed test tube, wall thickness 1.5 mm., inside diameter 21.5 mm., and height 20 cm.

Chamber V.—Soft glass hemispherical bottomed test tube, wall thickness 1 mm., inside diameter 22 mm., and height 15 cm.

Table II indicates that the cellulose acetate (I) and brass diaphragm (II) chambers were superior to the three test tubes as exposure chambers for emulsification. It was observed during exposure that in neither container was a well-defined system

TABLE I.—THE EFFECT OF ULTRASONICS AT 300 KC. UPON VISCOSITY OF EMULSIFYING AGENTS[a]

Emulsifying Agent	Initial Viscosity Centipoises	7½ Min. Viscosity Centipoises	15 Min. Viscosity Centipoises	30 Min. Viscosity Centipoises
Polysorbate 80, 25% v/v	15.3	15.3	15.3	15.3
Polyethylene glycol 400 monostearate 1.3% w/v	28.6	25.5	18.0	10.1
Tragacanth 0.8% w/v	5.7	3.2	2.6	2.2
Acacia 8% w/v	4.9	4.9	4.9	4.9
Hard soap 16% w/v	664.0	596.8	582.2	535.4
Sodium lauryl sulfate 20% w/v	104.4	104.4	104.4	104.4

[a] Viscosities taken with Hoeppler Viscosimeter at 25°.

TABLE II.—EFFECT OF TYPE OF EXPOSURE CHAMBER IN EMULSIFICATION WITH ULTRASONICS

Chamber Number	Dilution for Count	*N*	*H*, millions	*D*, microns	*S*, sq. cm./ml.
I	1:1,000	964	3,856	0.79	7.58×10^4
	1:1,000	922	3,688	0.80	7.47×10^4
II	1:1,000	934	3,736	0.80	7.50×10^4
	1:1,000	967	3,868	0.79	7.59×10^4
III	1:1,000	587	2,348	0.93	6.43×10^4
	1:1,000	586	2,344	0.93	6.43×10^4
IV	1:250	1,116	1,116	1.20	4.90×10^4
	1:250	1,021	1,021	1.23	4.87×10^4
V	1:1,000	654	2,616	0.90	6.66×10^4
	1:1,000	701	2,804	0.88	6.82×10^4

of stationary (standing) waves visible as in the test tubes. Agitation and churning of the liquid was greater in Chambers I and II. The low intensity of transverse waves in the walls of the cellulose acetate and brass diaphragm containers was observed. The chambers could be held between the fingers during exposure without discomfort. With the three test tubes intense transverse vibrations in the walls of the tube made holding the container between the fingers impossible. Frictional forces between the glass and the skin created enough heat to burn.

Bondy and Sollner (9) have shown that with a system in which well defined constant stationary waves are set upon the walls of the vessel, with intense transverse vibrations, conditions for coagulation become optimum. Zones of coagulation are set up at the nodes and antinodes of the stationary waves on the walls of the container. The smaller the diameter of the tube the more conspicuous the phenomenon becomes. As a result of the data in Table II, the brass diaphragm container was selected as the exposure chamber for the investigation.

Effect of Orientation of Exposure Chamber Above the Crystal upon Emulsification.—Richards and Loomis (2) indicated the importance of orientation of the exposure chamber above the vibrating crystal. By lowering a beaker of water by means of a micrometer screw and exposing it to ultrasonic vibrations at several points in its descent they found a maximum temperature rise at points where an exact number of half-wavelengths separated the bottom of the beaker and the quartz crystal. At these points, where the bottom of the beaker was in resonance with the waves traveling from the surface of the crystal, the greatest amount of energy entered the beaker.

To determine the effect of orientation of the ex-

TABLE III.—EFFECT OF ORIENTATION OF EXPOSURE CHAMBER ABOVE THE CRYSTAL UPON EMULSIFICATION

Sample Number	Distance Above Crystal, mm.	*N*	*H*, millions	*D*, microns
1	33.50	1,062	4,248	0.77
2	33.25	734	2,936	0.87
3	33.00	621	2,484	0.92
4	32.75	637	2,548	0.91
5	32.50	647	2,588	0.90
6	32.25	643	2,572	0.91
7	32.00	700	2,800	0.88
8	31.75	793	3,172	0.84
9	31.50	770	3,080	0.85
10	31.25	975	3,900	0.79
11	31.00	1,047	4,188	0.77
12	30.75	772	3,088	0.85
13	30.50	767	3,068	0.85
14	30.25	736	2,944	0.87
15	30.00	872	3,488	0.82
16	29.75	758	3,032	0.86
17	29.50	846	3,384	0.83
18	29.25	884	3,536	0.82
19	29.00	797	3,188	0.84
20	28.75	930	3,720	0.80
21	28.50	1,010	4,040	0.78
22	28.25	918	3,672	0.80
23	28.00	913	3,652	0.81
24	27.75	865	3,460	0.82
25	27.50	824	2,296	0.83
26	27.25	683	2,732	0.89
27	27.00	575	2,300	0.94
28	26.75	552	2,208	0.95
29	26.50	602	2,408	0.93
30	26.25	668	2,672	0.89
31	26.00	882	3,528	0.82
32	25.75	900	3,600	0.81

Dilution for globule count 1:1,000; V-0.001 cu. mm.

posure chamber above the crystal upon emulsification in the present investigation a micrometer screw was attached to the clamp holding the exposure chamber. Starting with a distance of 33.5 mm. between the brass diaphragm of the exposure chamber and the crystal face, the container was lowered 0.25 mm. in each step. At each 0.25-mm. step the bath was brought to 25° and a 25% light liquid petrolatum emulsion was made with 1% hard soap at a frequency of 300 kc., exposure time ten minutes, and plate current 200 ma. (approximately 95 w. acoustical energy) (Table III).

Since the velocity of sound waves in water is 1.43×10^5 cm./sec., from the formula:

$$\lambda = \frac{c}{f} = \frac{1.43 \times 10^5}{300{,}000} = 0.477 \text{ cm. or } 4.77 \text{ mm. wavelength of 300 kc. waves in water}$$

where

f = frequency, cycles/sec.
c = velocity in water, cm./sec.
λ = wavelength, cm.

Therefore a half-wavelength would be approximately 2.38 mm.

From Table III it can be seen that there is a rise in particle count and a decrease in "root mean cube diameter" at points approximately a half-wavelength apart in the lowering of the exposure chamber toward the crystal. This would indicate that at those points where the system was tuned the greatest amount of acoustic energy entered the exposure chamber.

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A Study of Emulsification with Ultrasonic Waves II.*

By HAROLD M. BEAL† and DONALD M. SKAUEN

Some factors influencing emulsification by the application of ultrasonic energy such as temperature and time of exposure are described. The emulsifying agents employed, listed in order of effectiveness, include hard soap, polyethylene glycol 400 monostearate U. S. P., polysorbate 80 U. S. P., acacia U. S. P., sodium lauryl sulfate U. S. P., and tragacanth U. S. P.

IN A PREVIOUS PAPER (1) were reported some of the results obtained in emulsification with ultrasonic waves.

In the present investigation four oils were selected for emulsification; Nujol,[1] light liquid petrolatum U. S. P.,[2] cottonseed oil U. S. P.,[3] and rectified turpentine oil N. F. Six emulsifying agents were used: Acacia U. S. P., tragacanth U. S. P., hard soap U. S. P., sodium lauryl sulfate U. S. P.,[4] polyethylene glycol 400 monostearate U. S. P. and polysorbate 80 U. S. P.[5]

Polysorbate 80 and polyethylene glycol 400 monostearate were selected as typical of the nonionic group of emulsifying agents. As anionic agents hard soap was chosen because of its long use and sodium lauryl sulfate as representative of the newer group of sulfated fatty alcohols. Tragacanth and acacia are the best known of the gums and were selected because of their wide use in emulsification.

The General Electric G-3 ultrasonic generator and the brass diaphragm exposure chamber described in the previous paper (1) were used in this investigation.

EXPERIMENTAL

The Effect of Temperature upon Emulsification with Ultrasonic Waves.—Ultrasonic waves have a very pronounced heating effect upon liquids (2). In emulsification with ultrasound there is heating at the oil/water interface; with a greater temperature rise with increased dispersion of the oil (and the resulting increased interface). Heating also takes place due to frictional forces between the liquid and the transversely vibrating walls of the container.

* Received March 31, 1955, from the University of Connecticut School of Pharmacy, Storrs.
The paper is based on a dissertation submitted by Harold M. Beal to the Graduate School of the University of Connecticut in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
Presented to the Scientific Section, A. PH. A., Boston meeting, August 1954.
† Present address: College of Pharmacy, University of Florida, Gainesville.
[1] Heavy Liquid Petrolatum, Esso Standard Oil Co., specific gravity @ 25°—0.8795, viscosity @ 25°—152.6 centipoises.
[2] Specific gravity @ 25°—0.8479, viscosity @ 25°—26.1 centipoises.
[3] Specific gravity @ 25°—0.9149, viscosity @ 25°—57.8 centipoises.
[4] Duponol® C, E. I. du Pont de Nemours & Co., Wilmington, Del.
[5] Tween® 80, Atlas Powder Co., Wilmington, Del.

To determine the effect of temperature upon emulsification with ultrasound it was decided to make a series of emulsions with and without cooling of the bath in the transducer assembly. To control the temperature a copper coil was placed at the perimeter of the sound chamber and cold water circulated through the coil. Temperature control was not complete but a rise of not more than 20° was achieved. In ultrasonic treatment of liquids isothermal conditions are impossible to attain even with the most efficient cooling system.

Emulsions of Nujol, light liquid petrolatum, cottonseed oil, and rectified turpentine oil were made, using 1% polyethylene glycol 400 monostearate as the emulsifying agent. Exposure times consisted of three, five, seven and a half, ten, fifteen, twenty, and thirty minutes; exposure distance 33.5 mm.; plate current 200 ma. (approximately 95 w. acoustical energy); and frequency 300 kc. (Fig. 1).

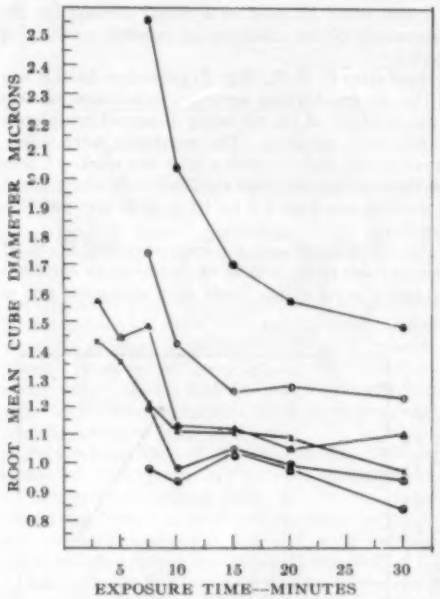

Fig. 1.—Effect of temperature upon emulsification with ultrasonic waves and polyethylene glycol 400 monostearate U. S. P.

●—Nujol—with cooling; ○—Nujol—without cooling; △—cottonseed oil—with cooling; ×—cottonseed oil—without cooling; ◍—light liquid petrolatum—with cooling; ◒—light liquid petrolatum—without cooling.

It was noted that rectified turpentine oil, which normally emulsified the easiest of the four oils, did not form an acceptable emulsion with polyethylene glycol 400 monostearate. At the end of exposure the emulsion had a curdled appearance and gradually separated into a three-layer system of oil, a poorly emulsified layer and an aqueous layer.

The relationship between the viscosity of the oil and the degree of dispersion was also quite evident. Audouin and Levavasseur (3) indicated that viscosity played an undeniable role in emulsification with ultrasonics, the less viscous oils being more easily emulsified. Such substances as xylene, toluene, etc., can be easily emulsified, even without emulsifying agents. Figure 1 indicates that the less viscous the oil the greater is the dispersion.

It was observed in this series of emulsions that cooling during exposure to ultrasonic waves did not increase the dispersion of the oil, as had been suggested by the literature. In this investigation, however, a protected emulsion was being considered whereas references in the literature often concerned emulsification without emulsifying agents. In the present investigation it was found that those emulsions which were cooled during formation were consistently less evenly grained and showed globules with equal or larger "root mean cube diameter" (1) than emulsions made without cooling. Since less viscous oils were more easily emulsified it was concluded that lowering the viscosity of the oil with the higher temperature favored greater dispersion.

Throughout this investigation it was apparent that the nature of the oil, excluding viscosity, also plays a role in the ease of emulsification with ultrasonic waves. In this series, for example, the three- and five-minute samples of Nujol and light liquid petrolatum were incompletely emulsified while samples of cottonseed oil, exposed for the same intervals, were acceptable emulsions.

The Effect of Time of Exposure upon Emulsification with Ultrasonic Waves.—It has been stated (4) that a minimum intensity of ultrasonic energy of the order of 10 w./cm.^{2} is necessary to produce emulsification. When this minimum energy value is passed the rate of emulsification increases as the acoustic energy available in the sound chamber is increased.

Bondy and Sollner (5) reported that after emulsification is complete continued exposure to ultrasonic waves leads to an increase in particle size in spite of the presence of an emulsifying agent. This would indicate that the length of exposure would be quite important in order to obtain minimum particle size.

With exposure times of three, five, seven and a half, ten, fifteen, twenty, and thirty minutes, emulsions of the four oils were made, using 1% hard soap as the emulsifying agent. Exposure distance between the brass diaphragm and the crystal was 35.5 mm., plate current 200 ma. and frequency 300 kc. (95 w. acoustical energy). By means of a cooling coil in the sound chamber of the transducer assembly, the bath was brought to 25° at the start of each exposure. Figure 2 shows the result of this series.

Had there been available for this investigation a generator with a higher power output the exposure periods could have been shortened considerably. A maximum exposure time of thirty minutes was set in this investigation on the assumption that it would be a long enough period of time to furnish a comparison.

Figure 2 indicates a general decrease in "root mean cube diameter," results contrary to those anticipated. This was confirmed throughout the entire investigation; with increased exposure times, emulsions protected by emulsifying agents progressively showed globules of decreasing "root mean cube diameter," rather than a tendency toward increased globule size. It is possible that longer exposure

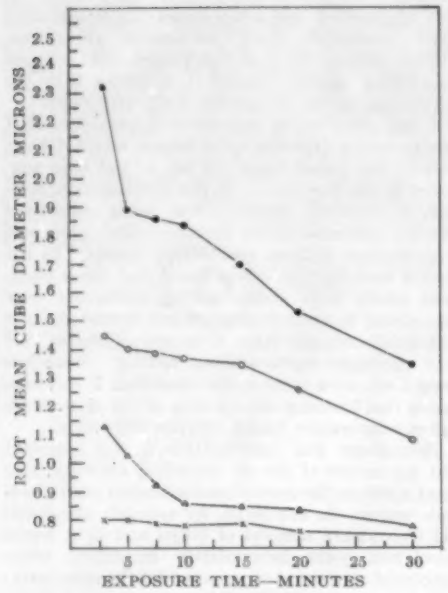

Fig. 2.—Effect of time of exposure to ultrasonic waves upon emulsification with hard soap U. S. P.

●—Nujol; ○—cottonseed oil; △—light liquid petrolatum; ×—rectified turpentine oil.

times could lead to an increase in globule size but up to thirty minutes this was not evidenced.

It is interesting to note from Fig. 2 that the "root mean cube diameter" is greater with increasing viscosity of the oils over the entire thirty-minute exposure range. Similarly, with the more viscous oils there was a greater variance between the three- and thirty-minute samples. It might be surmised that this was due to a lowering of viscosity of the oils due to temperature rise, to make for easier emulsification.

Comparison of Emulsions Made with Six Emulsifying Agents.—For comparison of emulsions made with the six agents used in the investigation the following general formula was used:

Oil	5.0 cc.
Emulsifying agent, *q. s.*	
Distilled water, *q. s. ad*	20.0 cc.

In the emulsions the following quantities of emulsifying agents were used: acacia, 6.25%; polysorbate 80, 0.5%; tragacanth, 0.625%; hard soap, 1%; polyethylene glycol 400 monostearate, 1%; and sodium lauryl sulfate, 0.5%. Exposure times were three, five, seven and a half, ten, fifteen, twenty, and thirty minutes, distance between the brass diaphragm and the crystal 33.5 mm., frequency 300 kc., and plate current 200 ma. (approximately 95 w. acoustical energy). By means of a cooling coil in the sound chamber of the transducer assembly, the bath was brought to 25° at the start of each exposure. Figures 1 through 5 show the results of the comparison.

In the emulsification process with ultrasonic energy the emulsion can be seen to go through quite definite stages. When the emulsion ingredients are placed in the exposure chamber and ultrasonic energy of sufficient intensity is applied there is first a disturbance of the surfaces of the two immiscible layers. Next the whole system is set into motion and there is a rolling and mixing of the oil and aqueous layers. Gradually the entire liquid becomes more and more turbid as the oil becomes emulsified, with increased motion of the chamber contents. Small globules of unemulsified oil can be seen circulating throughout the liquid. When all of the oil has been dispersed to particles smaller than macroscopic size, droplets of emulsion are thrown much higher above the liquid surface in larger amounts than previously and the walls of the vessel are covered with the emulsion. The completion of emulsification can be determined by this phenomenon. This was quite dramatic in emulsification of oils colored bright red with one-half grain of Sudan IV per liter of oil. When emulsification was complete the chamber contents assumed a faint pink color. The rate at which the above proceeds could be used as a visual method for the comparison of the efficiency of various emulsifying agents.

Hard soap U. S. P. (Fig. 2) proved to be the best of the six emulsifying agents. Emulsification was rapid, with all of the oil being dispersed completely within three minutes. The emulsions were stable, showing very little creaming after one week. Under the microscope the soap emulsions showed a range of particle size from 0.2 to 10 μ with few particles over 5 μ.

Polyethylene glycol 400 monostearate U. S. P. showed itself to be next in efficiency as an emulsifying agent in emulsions made with ultrasonic waves.

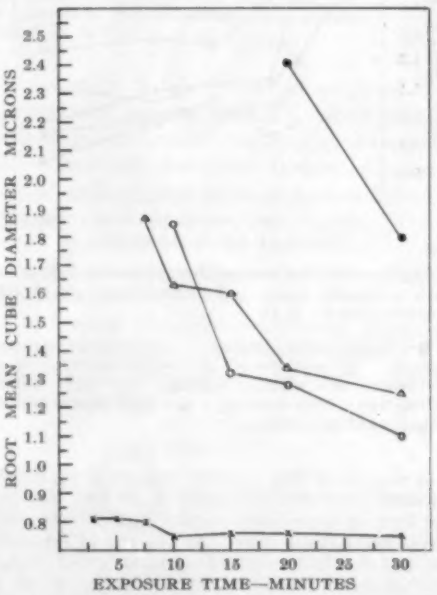

Fig. 3.—Emulsification with ultrasonic waves and sodium lauryl sulfate U. S. P.

●—Nujol; ○—cottonseed oil; △—light liquid petrolatum; ×—rectified turpentine oil.

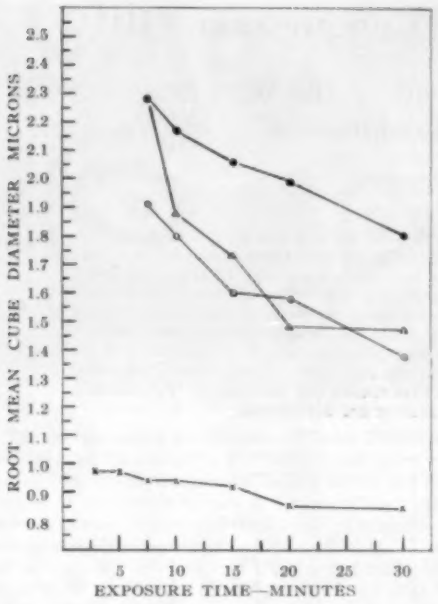

Fig. 4.—Emulsification with ultrasonic waves and acacia U. S. P.

●—Nujol; ○—cottonseed oil; △—light liquid petrolatum; ×—rectified turpentine oil.

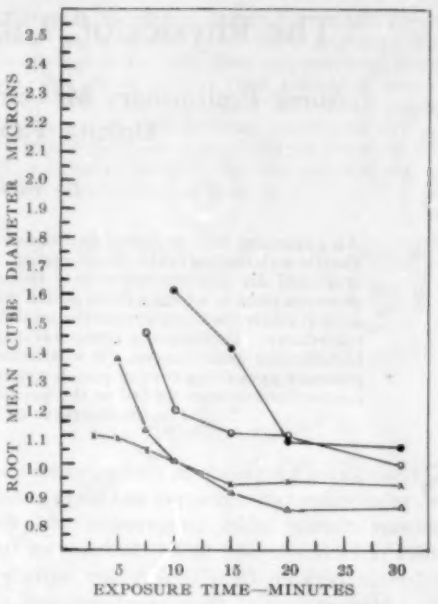

Fig. 5.—Emulsification with ultrasonic waves and polysorbate 80 U. S. P.

●—Nujol; ○—cottonseed oil; △—light liquid petrolatum; ×—rectified turpentine oil.

Its emulsions were comparable in appearance to the soap emulsions. Both these and the soap emulsions were stable after three months of shelf storage although those polyethylene glycol 400 monostearate protected emulsions with the shorter time exposures were highly creamed with a layer of emulsion and a clear layer below (Fig. 1).

Polysorbate 80 U. S. P. was found to be third in order of ability to protect an emulsion made with ultrasonic waves (Fig. 5). With Nujol it would not form a satisfactory emulsion in less than ten minutes' exposure time and with cottonseed oil in less than seven and one-half minutes.

Acacia U. S. P. (Fig. 4) worked moderately well as an emulsifying agent in this investigation. With the exception of the rectified turpentine oil emulsions the "root mean cube diameter" of its emulsions was greater than that of emulsions made with the three emulsifying agents discussed previously. The acacia emulsions were stable, but showed rapid creaming rates, separating into two distinct layers within a week.

Nujol emulsified poorly with sodium lauryl sulfate U. S. P. (Fig. 3). Only the twenty- and thirty-minute samples completely emulsified. Its emulsions of light liquid petrolatum, cottonseed oil, and rectified turpentine oil compared with those of acacia. Creaming rate was high; within a week two distinct layers formed.

Emulsions made with tragacanth U. S. P. were very poorly formed. With all four oils the product could hardly be considered an emulsion. A mixture formed with the appearance of an emulsion but within twenty-four hours it had separated into three layers; oil, a partially emulsified layer and a clear aqueous layer. This undoubtedly can be explained by the fact that tragacanth emulsifies due to the viscosity that it imparts to the emulsion. Since the viscosity of tragacanth mucilage is markedly lowered (1) by ultrasonic waves it no longer functions well as an emulsifier.

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The Physics of Tablet Compression VIII*

Some Preliminary Measurements of Die Wall Pressure During Tablet Compression

By EINO NELSON

An apparatus was designed and constructed to measure the pressure transmitted to the die wall during tablet compression. Essentially, the apparatus consisted of a conventional die constructed with a rectangular hole perpendicular to the axial compression hole in which a third punch was inserted and the necessary mechanical linkages to allow the force appearing on this punch to be transmitted to a force-measuring transducer. Preliminary compressions were made using sulfathiazole and sodium bicarbonate granulations. It was found that in both cases about 30 per cent of the pressure appearing on the punch appeared on the die wall. It was also found that magnesium stearate added to the granulations increased the percentage transmitted. The implications of these results are discussed.

STUDIES have been made on the magnitude and relationship between upper and lower punch pressure during tablet compression and the effect of lubricants and tablet thickness on the difference between these compression variables (1). Measurement of these quantities gave no information on the magnitude of the pressure which had its reaction on the die wall since there was no way to predict how closely compressed solids behaved as fluids. The purpose of this work is to describe the instrumentation accomplished to measure die wall pressure and report some preliminary results on its magnitude and the relationship it has to lower punch pressure. It was also desired to determine the effect of magnesium stearate in several concentrations on the relationship when compressing a sulfathiazole granulation and a sodium bicarbonate granulation.

EXPERIMENTAL

Apparatus.—The apparatus used to make the measurements is shown in Fig. 1. The assembly shown was fastened between the jaws of a high force hydraulic press and so aligned that when the press was activated, the upper punch moved into the die. As implied in the caption of this figure, the variables of compression measurable were upper punch pressure, lower punch pressure and pressure normal to the direction of compression or die wall pressure. The load cell used to measure die wall pressure was a Baldwin Type C, 10,000-pound total force unit. It was arranged so that the pressure appearing on the wall of the die as determined by the normal punch inserted in the wall of the special die was transmitted directly to the load cell's loading button. The force on the load cell was recorded on a Sanborn Magnetic Oscillograph in the same manner as previously described (2). The equipment used to measure upper and lower punch pressures in addition to the load cells were galvanometers and associated electrical components. A complete description of the hydraulic press and components for measuring upper and lower punch pressures as well as upper punch displacement will be described in a forthcoming publication (3).

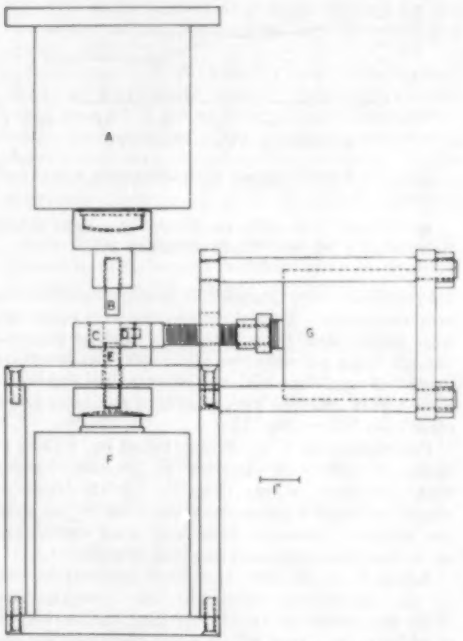

Fig. 1.—Assembly to measure die wall pressure.

The details of the special punch and die assembly for carrying out the compressions in which die wall pressure was determined is shown in Fig. 2. The special die, $1^{1}/_{16}$ inches in diameter and $^{9}/_{16}$ inches long, was made with a rectangular $^{3}/_{16}$ by $^{1}/_{4}$-inch hole in the center of the wall perpendicular to a $^{3}/_{8}$-inch hole drilled axially through the center of the die. The normal punch was closely fitted to the rectangular hole and faced on the end extending into the axial $^{3}/_{8}$-inch hole to the contour of this hole.

* Received February 5, 1955, from the Research Laboratories of the School of Pharmacy, University of Wisconsin, Madison 5.

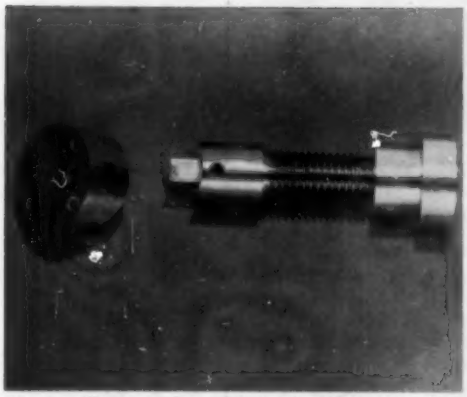

Fig. 2.—Close-up of special punch and die assembly.

The normal punch was held by a $^{5}/_{8}$-inch diameter rod threaded for about $^{2}/_{3}$ of its length and faced at the end in which the normal punch fitted to the contour of the outside surface of the special die. All parts were made from cold-rolled steel. The normal punch was face-hardened after machining. The normal punch when held in its holder and with the holder flush against the die was set to extend about 0.001 inch into the die to compensate partially for the small outward displacement of this punch during compressions. Lower and upper punches were $^{3}/_{8}$ inch in diameter and flat-faced.

The load cells and associated equipment were calibrated with known loads prior to this series of compressions. The normal punch and die assembly was assembled into the measuring unit under about 25 kilogram force by tightening the locking nut on the end of the holder which rested on the loading button of the load cell.

All compressions were made to a pressure of 2100 Kg./sq. cm. (about 30,000 pounds per square inch) on the upper and lower punches. Upper and lower punch pressures were essentially the same since prior to compressions the die wall was lubricated with magnesium stearate by dusting on a thin film of this material. Preliminary compressions were made to the stated pressure to determine the weight thickness ratio for the material under study. From the information obtained the weight needed to make a tablet nearly exactly as thick as the thickness of the normal punch was calculated. The calculated amount of material was weighed out and tamped into the die. This was necessary since the fill was always greater than the amount the die would hold when loosely packed. Before compressions, the lower punch was adjusted so that its face was just at the bottom edge of the normal punch.

Using the procedure just described, it was possible to form tablets within 0.002 inch in thickness of the thickness of the normal punch. At the end of compressions, then, the face of the upper punch was the the thickness of the normal punch above the face of the lower punch.

During compressions about 12 readings were taken of lower punch and die wall pressure between zero and maximum pressure on the lower punch. The hydraulic machine used to carry out the compressions was slow acting and the time required to make a compression was between twenty-five and forty-five minutes.

The sulfathiazole and sodium bicarbonate granulations were used as 20/30 mesh granules. The granulations were made from the powdered materials and starch paste. They contained, respectively, 5 and 2.5% partially hydrolyzed starch. The amount of each material compressed to meet the thickness requirement previously discussed was 500 mg. sulfathiazole granulation and 650 mg. of sodium bicarbonate granulation.

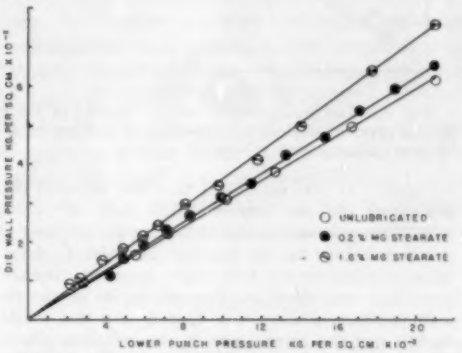

Fig. 3.—Die wall pressure as function of lower punch pressure during compression of sulfathiazole granulation.

RESULTS

Die Wall Pressure with Lubricated and Unlubricated Sulfathiazole Granulation.—The relation between die wall pressure and lower punch pressure found in the compression of sulfathiazole granulation is shown in the lower curve of Fig. 3. From the curve it appeared that about a constant 30% of the pressure on the lower punch appeared on the die wall. When 0.2% by weight of magnesium stearate was added to the granulation the percentage of lower punch pressure found on the die wall was increased slightly as shown by the curve immediately above the curve for unlubricated granulation in Fig. 3.

At 1.6% magnesium stearate on the granulation, the percentage of pressure transmitted was increased to about a constant 40% of lower punch pressure. The results obtained with 0.4 and 0.8% magnesium stearate on the granulation were not plotted since they coincided very nearly with the results obtained using 1.6%.

Die Wall Pressure With Sodium Bicarbonate Granulation.—When the sodium bicarbonate granulation was compressed the results shown graphically in Fig. 4 were obtained. A deviation from the linear relationship obtained in the compression of sulfathiazole occurred with both the lubricated and unlubricated granulation. The presence of 0.2% magnesium stearate caused a somewhat greater percentage of the applied pressure to be transmitted than occurred when the sulfathiazole granulation was compressed with the same amount of lubricant. The percentage transmission of pressure to die wall was nearly the same for both unlubricated granulations.

Effect of Extrusion During Compression.—It was observed that at the end of compressions a slight outward displacement of the normal punch had

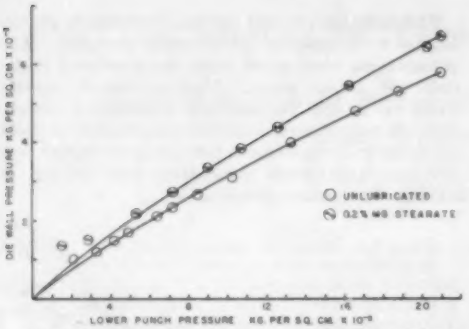

Fig. 4.—Die wall pressure as a function of lower punch pressure during compression of sodium bicarbonate granulation.

occurred. It was estimated that the outward displacement did not exceed 0.003 inch of which 0.001 inch was compensated for by initial inset of this punch into the die cavity. In order to determine whether or not this slight outward displacement had any effect on the magnitude of the die wall pressure measured, a special test was made. In this test the normal punch assembly was placed in the apparatus and prestressed by tightening the locking nut which rested on the loading button of the load cell, until the force on the load cell was equal to the force corresponding to the maximum die wall pressure observed with maximum lower punch pressure in the compression of unlubricated sulfathiazole granulation.

The die was filled with the correct amount of granulation and the lower punch pressure brought up to the maximum used in these compressions. The die wall pressure was then read and compared with the value obtained with the usual compression technique. The die wall pressure exceeded the value obtained before by about 15 Kg./sq. cm. Drift in zero setting of the magnetic oscillograph was about plus or minus 30 Kg./sq. cm. so the deviation found was well within the allowable error. In compressions to higher pressures the prestression technique should prove useful in case extrusion of compressed material into the normal punch hole becomes excessive.

DISCUSSION

The marked difference between die wall pressure and lower punch pressure indicates that the strength of bonding between granules in a tablet in planes parallel and normal to the direction of compression should not be the same. In Fig. 5, then, bonding in the plane ABCD normal to the direction of compression should be stronger than bonding in the plane WXYZ parallel to the direction of compression. The difference in bonding strength should be proportional to the difference in pressures measured on the lower punch and die wall.

It would probably be difficult to test this contention by tablet hardness tests. Any distortion in the shape of the granules as a consequence of compression would cause an irregularity of structure in the tablet.

The increase in die wall pressure observed when lubricant was added to the granules compressed showed that lubricant acted to decrease inter-

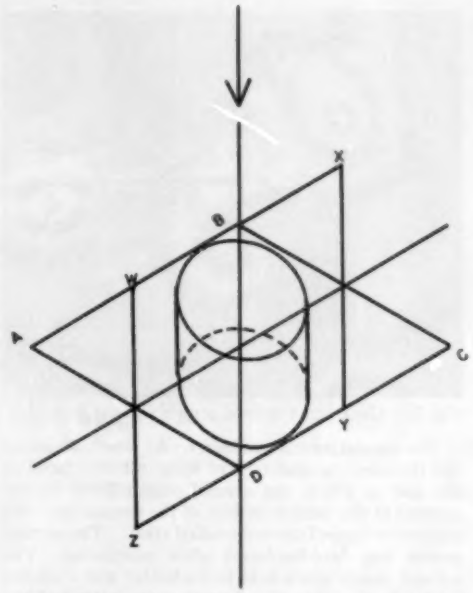

Fig. 5.—Bonding planes in a cylindrical tablet. Plane ABCD normal to direction of compression. Plane WXYZ parallel to direction of compression. Arrow indicates compression direction.

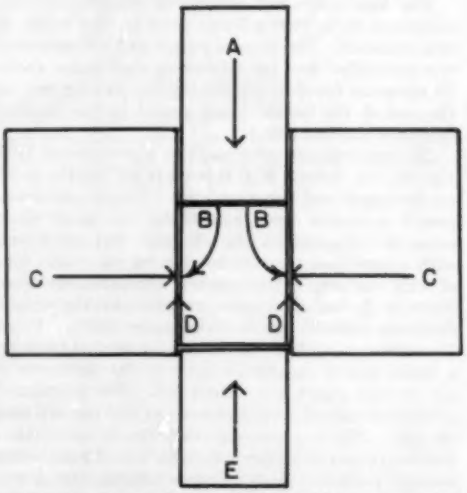

Fig. 6.—Pressure lines during tablet compression. *A*—upper punch pressure line, *B*—pressure lines to die wall, *C*—die wall pressure lines in reaction to *B*, *D*—die wall friction pressure lines, and *E*—lower punch pressure line.

particle friction. It has been previously shown that efficient lubricants added to tablet granulations caused a marked reduction in the difference between upper and lower punch pressure (1). The same effect could be obtained by using lubricant on the die wall only. In all the compressions reported in this paper the die wall was so lubricated yet when lubricant was also added to the granulation the

percentage of pressure exerted that reached the die wall increased. It appeared then, that in addition to acting on the die wall to reduce the difference between upper and lower punch pressure, lubricant also acted to tend to equalize pressure distribution in the compressed granules. It may be possible that with other lubricants the degree of equalization will be greater.

The distribution of pressures in a die cavity during compression in the single punch process is shown in Fig. 6. Upper punch pressure that had reactions, *C*, on the die wall via force lines, *B*, resisted to a certain extent further downward movement of this punch. The amount of resistance, *D*, offered was the measured difference in upper and lower punch pressures. For a given tablet, the difference between upper and lower punch pressure depends only on the coefficient of friction between the die wall and tablet and on the pressure that is transmitted to the die wall. These quantities are related by the following expression:

$$\Delta P = \mu Pw$$

where ΔP is the pressure difference, μ the coefficient of friction and Pw is the pressure transmitted to the die wall. Since lubricant added to granulation increased the pressure on the die wall it should be more efficient if added only to the die wall in so far as reduction of the difference between upper and lower punch pressure is concerned.

CONCLUSIONS

1. Pressure on the die wall can be successfully measured by means of the apparatus described in this paper.

2. Pressure distribution is not uniform in the die cavity during tablet compression although pressence of lubricants on granules tends to equalize the distribution.

3. As a consequence of this inequality of pressure distribution in compressed tablet formation, bonding in planes parallel to the compression direction should be weaker than bonding in planes normal to the compression direction.

4. Due to the increase in die wall pressure experienced with lubricated granules, a tablet lubricant should be more efficient if added to the die wall only in so far as reduction in difference between upper and lower punch pressures is concerned.

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The Pharmacologic Effects of a New Analgesic α-4-dimethylamino-1,2-diphenyl-3-methyl-4-propionyloxybutane*

By E. BROWN ROBBINS

The results of pharmacologic studies with a new analgesic compound are presented. They show analgesic activity in animals with a notable lack of tolerance development and/or respiratory depression.

IN CLINICAL PRACTICE, potent analgesic activity has invariably been associated with certain undesirable side reactions. The most objectionable of these are the development of tolerance which presages addictive liability, and respiratory depression which has serious consequences in general anesthesia and obstetrics. In a search for analgesic drugs which might be relatively free from these side reactions, Pohland and Sullivan (1) submitted a series of four compounds which in screening tests appeared to be promising. One of these, No. 16298, has been subjected to a more extensive study than the others in the hope that the desirable properties might hold in man.

Chemically this compound is α-4-dimethylamino-1,2-diphenyl-3-methyl-4-propionyloxybutane and belongs to a series of esters of 4-dialkylamino-1,2-diphenyl-2-butanols (1).

METHODS

A modification of the Davies (2) technique for measuring pain threshold in rats was used. The painful stimulus was provided by a heated resistance-wire built into a water-cooled tail rest and curved so that the part of the tail exposed was approximately $^1/_{16}$ inch from the wire. An untreated animal whose tail was placed in this device would attempt to remove it within three to five seconds. Any significant prolongation of this reaction time in groups of treated rats was considered indicative of an elevation of the pain threshold. An arbitrary cut-off time of fifteen seconds was adopted in order to avoid cutaneous damage.

* Received February 3, 1955, from the Lilly Research Laboratories, Eli Lilly and Company, Indianapolis 6, Ind.

The author gratefully wishes to acknowledge the assistance of Drs. K. K. Chen, F. G. Henderson, and R. C. Anderson and Messrs. R. H. Bills and W. L. Kurtz.

TABLE I.—REACTION TIME VARIATIONS AMONG AND WITHIN GROUPS

Rat No.	Rat Wt., Gms.	Dose cc./Kg. S.C.	Reaction Time in Seconds, After 30 Min.	After 60 Min.	Mean/Rat	Difference from Control
			Drug: Saline			
1	71		3	3.5		
2	67	5	2.5	2.5	3.38	
3	68		4	6		
4	67		2.5	3		
5	76		3	3		
6	65	5	2.5	2.5	3.38	
7	71		7	3.5		
8	73		2.5	3		
9	71		3	4		
10	70	5	3	3	3.81	
11	75		4	4.5		
12	77		5	4		
13	68		3	3		
14	72	5	2.5	3.5	3.56	
15	76		2.5	5		
16	67		5	4		
17	73		3.5	3		
18	75	5	3	3	3.94	
19	67		4	5.5		
20	76		3.5	6		
			Drug: No. 16298			
			mg./Kg., S. C.			
1	65		>15	6		
2	64	20	10	5	9.20	+5.38
3	69		8	>15		
4	73		8	6.5		
5	62		3.5	4		
6	61	5	5.5	6.5	4.32	+0.50
7	70		3	3		
8	62		4	5		
9	60		7	7		
10	58	40	>15	>15	12.70	+8.88
11	70		>15	>15		
12	67		>15	12.5		
13	70		8	4.5		
14	65	10	7.5	8	6.12	+2.30
15	65		6.5	7		
16	65		3	4.5		
17	62	Saline	2.5	3		
18	65	Controls	4	3.5	3.82	
19	67	5 cc./Kg., S. C.	3.5	3		
20	70		5.5	5.5		

All Controls

Twenty rats, in five groups of four, were used in each test. Appropriate concentrations of compounds to be tested were placed in five bottles, unlabeled except for numbers to correspond to the five groups. When equal volumes per unit of body weight were given, the desired dose levels resulted. At least one of the five bottles always contained physiological saline to give a baseline response, and the operators were never aware of the bottle contents. Pain thresholds were usually read at thirty minutes and one hour following a subcutaneous injection and expressed as the mean in seconds of eight reaction times for a group of four rats.

In order to determine the variation among and within groups, from time to time all five bottles were filled with saline. An example of one of these as well as one wherein various doses of No. 16298 were given are shown in Table I. By these and other data it was shown statistically that a mean difference in the reaction times between treated and control groups of one and two-tenths seconds per rat could be considered significant. In our experience morphine, methadone, and other drugs of this type have increased the reaction time to the limit of fifteen seconds (ten to twelve seconds per rat) in doses that produce no other abnormal signs. The most potent of the antipyretics, on the other hand, were capable of only two and a half to three seconds increase when doses just below the toxic level were used.

A modification (3) of the Hardy-Wolff technique (4) for measuring pain threshold was used on dogs.

In anesthetized dogs, minute volume was measured with an apparatus for continuous recording of the volume of expired air (5). Acute toxicity was determined as the LD_{50} following intravenous administration to albino mice. Subacute toxicity was tested in two groups of five rats. One group received 40 mg./Kg. and the other 80 mg./Kg. daily by subcutaneous route for one month.

An attempt was made to demonstrate tolerance to the analgesic effect in 20 rats by first determining the analgesic activity of 10 and 20 mg./Kg. injected subcutaneously and then giving 40 mg./Kg. twice daily for nine days after which pain threshold changes were again recorded following the original doses of 10 and 20 mg./Kg. Two dogs were placed on the following subcutaneous dosage program in an attempt to induce physical dependence: 10 mg./Kg. for two days, 20 mg./Kg. for four days, 20 mg./Kg. twice daily for three days, 30 mg./Kg. twice daily for two days, and finally 40 mg./Kg. twice daily for three days. In an attempt to demonstrate acute tolerance to high doses (6), 10 rats were given 200 mg./Kg. subcutaneously and one hour later five were given 5 mg./Kg. of N-allylnormorphine intravenously.

The constipating effect was studied in rabbits. The number of scybala passed in six hours was counted following subcutaneous doses of 20 and 40 mg./Kg. Intestinal motility was recorded in anesthetized dogs with a balloon-tambour arrangement. Anti-inflammatory activity was tested in rats that had a chemically induced inflammation of the ankle.

RESULTS

Analgesia.—A significant elevation of pain threshold occurred in rats following subcutaneous doses of 5 mg./Kg. of No. 16298; doses of 10 to 20 mg./Kg. compared favorably with 1 mg./Kg. of methadone hydrochloride and 20 to 40 mg./Kg. was equivalent to 2 mg./Kg. of methadone, which produced a profound analgesia as judged by this method.

In two groups given 10 mg./Kg., and four groups given 20 mg./Kg. by mouth the mean increases in reaction times were 1.070 and 3.257 seconds, respectively. These figures were so similar to those for 5 and 10 mg./Kg. by subcutaneous route (Table II) that No. 16298 was concluded to be about one-half as potent by mouth as by parenteral route.

TABLE II.—MEAN INCREASES IN REACTION TIMES OVER CONTROL REACTION TIMES FOR GROUPS OF 4 RATS GIVEN VARIOUS SUBCUTANEOUS DOSES OF No. 16298[a]

5 mg./Kg.	10 mg./Kg.		20 mg./Kg.	
1.12	2.82	4.00	6.62	6.80
2.20	3.82	4.50	8.82	5.35
0.82	0.25	4.75	6.82	3.85
1.32	4.40	5.25	8.88	6.82
	1.02	3.58	7.45	5.08
	2.48	2.28	5.08	6.58
	3.20	0.65	7.75	5.62
	1.82	1.22	8.62	2.70
	2.58	1.20	8.42	9.25
	5.25	1.20	9.95	12.00
	7.45	5.12	8.82	
	6.50	3.30		
Mean ± S.E.:				
1.365 ± .230	3.277 ± .388		7.204 ± .473	

[a] (See column "Difference from control" in Table I).

A direct comparison by this method of compound No. 16298 with codeine, administered subcutaneously, showed both drugs to be approximately equal in potency and duration of action on a weight for weight basis (Table III). In this study pain thresholds were read at one, one and a half, two, and three hours in order to perceive any difference in duration of action. The peak of activity for both drugs occurred at sixty to ninety minutes so the readings at two and three hours have the effect of reducing the mean figures as compared to those for similar doses of No. 16298 in Table II.

In four trained dogs the subcutaneous injection of 2 to 4 mg./Kg. of compound No. 16298 was ineffective, whereas 10 mg./Kg. significantly raised the skin twitch threshold.

TABLE III.—COMPARISON OF No. 16298 WITH CODEINE SUBCUTANEOUSLY FOR ANALGESIC ACTIVITY AND DURATION OF ACTION IN RATS[a]

No. 16298		Codeine	
10 mg./Kg.	20 mg./Kg.	10 mg./Kg.	20 mg./Kg
1.08	6.18	5.25	9.12
0.88	7.68	2.25	5.30
1.80	5.25	7.25	0.00
	0.60	1.68	2.00
	2.05	4.82	6.30
Mean:			
1.250	4.350	4.250	4.545

[a] Figures are mean increases in reaction times over those of control groups for groups of 4 rats.

Respiratory Effect.—In anesthetized dogs, the minute volume was not reduced by sublethal intravenous or subcutaneous doses. In two dogs given an intravenous infusion of No. 16298 at the rate of 0.5 mg./Kg. per minute until death, respiration was not depressed until 80–90% of the total dose had been given; death was due, however, to respiratory arrest.

Toxicity.—The intravenous LD_{50} of No. 16298 in mice was 34.7 ± 1.7 mg./Kg. (Table IV).

TABLE IV.—LD_{50} DATA FOR No. 16298 I. V. IN MICE

Dose mg./Kg.	No. Dead/ No. Injected	LD_{50} ± S. E.
30	1/5	
33	3/10	34.7 ± 1.7
36.5	7/10	

In the subacute studies the mean weight gain per rat in the group that received 40 mg./Kg. daily was 61.6 Gm. and, in the group given 80 mg./Kg. daily, 57.0 Gm. One rat in the second group died after seven doses; hematuria was present but the cause of death was not apparent. Survivors were sacrificed at the end of the test period and submitted for necropsy. The fact that all tissues as well as red cell, white cell, and differential counts were normal, justifies the conclusion that No. 16298 in daily doses of 80 mg./Kg. for one month did not cause visceral or hematological changes in rats.

Tolerance.—In the rats given 40 mg./Kg. twice daily for nine days there was no tolerance to the

analgesic acitivity of a 10 or 20 mg./Kg. dose. During the nine-day period any attempt to increase the dose from 40 to 80 mg./Kg. twice daily resulted in death, which would indicate a lack of tolerance to the toxic effects also.

The rats injected subcutaneously with 200 mg./Kg. of No. 16298, followed by 5 mg./Kg. of N-allylnormorphine intravenously one hour later, exhibited no signs of acute physical dependence. The toxic signs (salivation, convulsions, and rigidity) which were seen following this large dose of No. 16298 were prevented by the N-allylnormorphine.

Of the two dogs given increased daily and twice daily doses of No. 16298, one died on the day of the last increase; the cause of death was not apparent and no abnormal tissue was seen on gross or microscopic examination. The other dog was febrile the last five days. This may have been due to an infection caused by the repeated subcutaneous injections, for although reasonable care was taken to avoid sepsis, a slough occurred on the last day at one of the injection sites. An attempt was made to induce withdrawal signs with an intravenous dose of 1 mg./Kg. of N-allylnormorphine. The rectal temperature rose from 103.9 to 104.9°. The pupils remained normal in size and reacted to light. The pulse rate, which was 128 when N-allylnormorphine was given, rose to 175 an hour later, and fell to 120 after another hour. The animal showed slight ataxia. Food and water were accepted one hour, and again two hours, following the N-allylnormorphine. The respiration rate remained between 20 and 32 per minute. No more No. 16298 was given and the dog was observed for several days. The temperature returned to a range of 101.2–101.6°. No signs of the abstinence syndrome were seen.

Other Effects.—A slight increase in motility of the duodenum of dogs was observed following intravenous doses of 1 or 2 mg./Kg. Number 16298 appeared to be less active in this respect than equivalent doses of methadone.

Subcutaneous doses of 20 mg./Kg. had no constipating effect in rabbits; 40 mg./Kg. resulted in a reduction in the number of scybala, approximating that produced by 2 mg./Kg. of methadone.

No anti-inflammatory action was seen when No. 16298 was given to rats having a chemically induced inflammation of the ankle.

There were no pronounced changes in the electrocardiograms of the two dogs given the intravenous infusion in the respiratory studies. A slight decrease in rate occurred late in the course of the injection and the *T* wave, which was inverted initially in one dog and isoelectric in the other, changed to an upright *T* of slightly higher voltage than normal. It was concluded that No. 16298 had no deleterious effect on the heart.

Discussion.—The tolerance studies are only preliminary and of but two weeks' duration. They do, however, indicate that, in contrast to narcotic analgesics, No. 16298 fails to produce tolerance to its analgesic and toxic effects. It is hoped that these findings may indicate a very low addictive liability on clinical trials.

The ability of No. 16298 to produce profound analgesia in animals without depressing respiration is also an encouraging sign.

SUMMARY

A new compound, α-4-dimethylamino-1,2-diphenyl-3-methyl-4-propionyloxybutane, was studied for its analgesic activity and side reactions in animals.

This product had an analgesic activity in rats equal to codeine on a weight for weight basis, and was capable of producing a profound morphine-type analgesia in these animals.

No respiratory depression was seen in dogs until near lethal doses were given.

Attempts to induce tolerance to analgesic or toxic manifestations in two weeks were unsuccessful in rats and dogs.

No deleterious cardiac changes were seen in anesthetized dogs following slow intravenous infusion of near lethal doses.

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Rapid Surface Tension Determinations Using a Modified Pendant Drop Technique*

By J. A. CAMPBELL, J. E. CHRISTIAN, and J. R. EATON

In the search for a way of speeding up the pendant drop method of measuring surface tension, a development was made which permitted a reduction of approximately 75 per cent in time required for a determination. This modified method permits a monetary saving in addition to the saving in time, by eliminating a photographic process. This method offers advantages over other surface tension methods for certain uses.

THE PENDANT DROP METHOD has certain advantages over other methods of measuring surface tension. One important advantage is that only a few drops of the liquid are required for a determination. It can also be used advantageously to study a rate of change in surface tension over a period of time without disturbing the surface of the liquid. It is not claimed that the pendant drop method is superior to all others for all conditions and circumstances, because the nature and purpose of a determination would dictate the choice of method for a particular situation. However, certain characteristics of the pendant drop method make it the method of choice for many uses.

Andreas, Hauser, and Tucker (1) made a critical study of the pendant drop method for measuring surface tension. A special apparatus was constructed for the determinations with the idea of controlling as many of the variable factors as possible. They found that when certain factors are controlled, such as evaporation and temperature, a high degree of accuracy can be obtained.

The greatest obstacle to extensive application of the pendant drop method is the unusually great amount of time required for a single determination using the conventional way of photographing the pendant drop and measuring the dimensions from the image obtained. It appeared that the best approach to this problem of time consumption was to attempt to eliminate the photographic process. By finding some method of obtaining the dimensions of the drop directly, the photography could be eliminated, and thereby a major saving in time could be brought about.

EXPERIMENTAL

With the objective of devising some method of obtaining the dimensions of the pendant drop by direct measurement, an instrument was developed which was called the tangeometer. The tangeometer fulfilled all expectations in accomplishing the role for which it was intended. Experiments showed that data thus obtained compared favorably with that obtained under the same conditions using the conventional photographic method.

The tangeometer is based upon the idea of projecting the image of a pendant drop upon a screen to which is attached an adjustable square for the purpose of measuring the dimensions of the drop (Fig. 1). The square is adjusted around the image

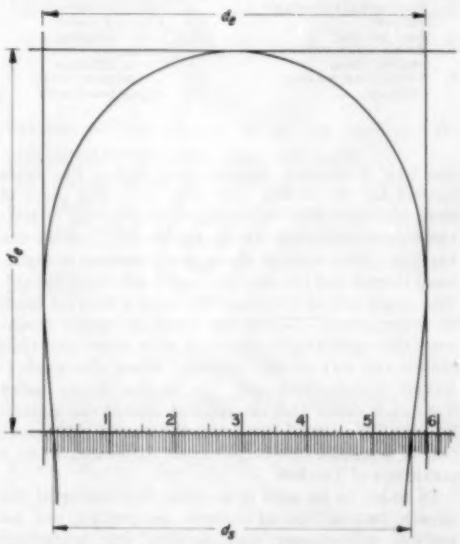

Fig. 1.—Drop image enclosed by tangeometer hairlines and scale with dimensions indicated.

of the drop so that three sides of the square are tangent to three sides of the drop. In order to calculate surface tension from the dimensions of a drop d_e, or equatorial diameter, is first measured; then d_s, or selected diameter, is measured at a distance from the tip of the drop equal to d_s. As can be seen in Fig. 1, d_e is equivalent to one side of the square, and the distance from the tip of the drop to d_s is equivalent to a second side of the square. By graduating the side of the square which falls on d_s, the value of d_e can be read from the scale.

The square is composed of two pairs of arms, each pair forming a right angle. The angle formed by

* Received August 28, 1954 from the School of Pharmacy and the School of Electrical Engineering, Purdue University, West Lafayette, Ind.

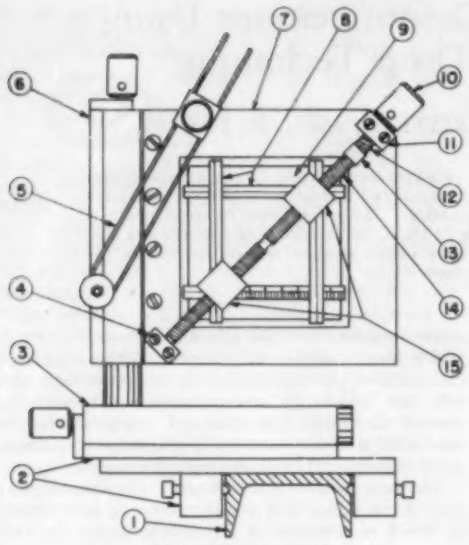

Fig. 2.—Tangeometer

1. Apparatus bench
2. Saddle and base
3. Horizontal adjustable support
4. End bearing
5. Swing-away parallax reading glass
6. Vertical adjustable support
7. Frame
8. Lucite strips
9. Image screen
10. Knurled knob
11. Collar bearing
12. Spring
13. Spring retainer
14. Adjustment screw
15. Adjustment nuts

one pair is exactly opposite and facing the angle formed by the second pair (Fig. 2). The pairs of arms are anchored on opposite ends of an adjustment screw situated at an angle of 45° from the vertical. One end of the screw possesses a right-hand thread and the opposite end a left-hand thread. The upper end of the screw contains a knurled knob for adjustment. When the knob is turned clockwise, the right angles approach each other and thus reduce the size of the square. When the knob is turned counterclockwise, the angles move away from each other and expand the size of the square. By manipulation of the knob, the size of the square can be controlled at any desired diameter, up to a maximum of 3 inches.

In order to be able to position the center of the square, two additional controls are needed, one for vertical adjustment and another for horizontal adjustment. The vertical screw with knurled knob serves the purpose of adjusting the face of the instrument in a vertical position, and the horizontal screw with accompanying knurled head serves to move the face from left to right and vice versa.

By use of the three knobs, any adjustment can be made quickly and smoothly. The square can be made tangent to the image on three sides, and the dimensions read off quickly from the graduation on the fourth side.

To serve the twofold purpose of preventing parallax and providing magnification for reading the scale, an extra optical aid was added to the tangeometer. The swingaway parallax reading glass (Fig. 2) can be moved to any position on the screen and at the same time always maintain a vertical position in respect to the screen.

An optical glass water cell was adapted to serve the purpose of a chamber for enclosing the drop. The top of the cell was enclosed with a brass cap which was made to fit snugly into the opening. The cap contains holes into which fit brass holders for mounting the thermometer, the syringe for forming the pendant drop, and the injection tube.

A carbon arc illuminator for microprojection (Fig. 3) was adapted as an illumination source. This was found to serve excellently as an illumination source, as well as providing a condensing lens for producing parallel rays of light which add to the accuracy of measurement.

A magnifying lens assembly (Fig. 3) was mounted on the base just in front of the drop. The purpose of the lens assembly is to focus the drop image upon the ground-glass screen, after having been magnified to a size which can be comfortably measured.

A special cell holder and support (Fig. 3) were constructed to anchor the odor chamber in the constant temperature bath so that the pendant drop would always fall in the beam of light.

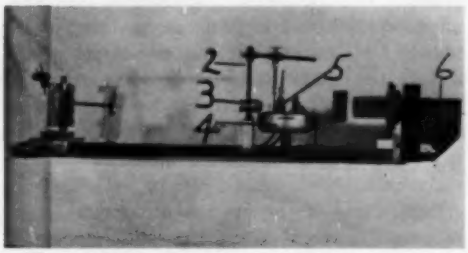

Fig. 3.—Tangeometer assembly. *1.* Drop measurement apparatus. *2.* Cell holder. *3.* Magnifying lens. *4.* Constant temperature bath. *5.* Chamber supported in bath. *6.* Carbon arc lamp.

The magnification of the drop is determined by the use of a small cylinder, the diameter of which is accurately known. The cylinder is suspended on the tip of a syringe in the same plane in which the drop would hang. The diameter of the cylinder, after magnification, is read on the screen from the centimeter scale. The magnification factor is obtained by dividing the actual diameter into the magnified image diameter.

The liquid, of which the surface tension is to be determined, is placed in a 1-ml. B. and D. tuberculin syringe, and the syringe placed in the proper receptacle on the top of the cell. A drop of the liquid is forced out of the tip of the syringe. With the temperature controlled at the proper degree by a constant temperature bath, the apparatus is ready for surface tension determinations to begin. Other liquids can be tested by simply removing the syringe and replacing with a similar syringe containing the liquid to be tested. A single determination can be made or, if desired, determinations at intervals over a period of time can be made.

Extreme care must be exercised to prevent errors due to contamination. The surface tension of most liquids is greatly altered by traces of contaminants. To minimize this error, certain procedures were followed. The syringe and cell were washed in

chromic acid cleansing mixture, then in distilled water, and followed with five minutes boiling in distilled water.

The data necessary for calculation of surface tension which must be read from the tangeometer screen (Fig. 1) are the equatorial diameter, d_E, and the selected diameter, d_S. The d_E value can be read directly, but d_S must be obtained indirectly. The point of intersection of the drop border with the right end of the graduated hairline is read and recorded. The difference between d_E and the intersection value is subtracted from the intersection value. The remainder is the value of d_S. The data are recorded and treated as shown in the following example (Table I).

TABLE I.—DETERMINATION OF SURFACE TENSION OF A DROP OF DISTILLED WATER[1]

T	dx	d_E	d_S	S	F	d_E in cm.	M	g	ρ_1	ρ_2	γ
25°	5.30	5.51	5.09	0.923	0.386	0.430	12.8	980	0.997	0.001	70.0

Where
- T = the temperature of the pendant drop.
- dx = the point where the drop intersects the hairline on the right end of the screen scale (Fig. 1).
- d_E = the maximum diameter of the image, as read from the screen scale (Fig. 1).
- d_S = the selected diameter which is obtained by subtracting dx from d_E, then subtracting the difference from the value dx.
- S = the ratio of the diameters, obtained by dividing d_S by d_E.
- F = a function of S which is obtained from a table of previously determined data (1) using the value S.
- d_E in cm. = the maximum diameter of the drop in cm., obtained by dividing d_E by the magnification value.
- M = magnification which is determined as previously explained.
- g = the acceleration of gravity, which is 980 cm. sec.$^{-2}$.
- ρ_1 = the density of the liquid forming the drop (in this example, water).
- ρ_2 = the density of the atmosphere surrounding the drop (in this case, air).
- γ = the surface tension in dynes/cm., which is obtained by use of the formula $\gamma = g(\rho_1 - \rho_2)d_E^2F$.

DISCUSSION

Even though the development of the tangeometer came about as a result of a search for an improved method of measuring odors, its application is by no means limited to this area alone. The successful performance in this field suggests many other applications where the advantages of this method can be successfully exploited. The method is particularly suited for the study of certain liquids which are available only in very limited quantities. A study of the aging of drops is a simple matter by the use of this method. Certain interfacial tension studies could be advantageously carried out through the utilization of this method.

[1] The data in Table I must be recorded and calculation made according to the formula for each surface tension determination.

SUMMARY

1. Studies were made to find a way of improving the pendant drop method of measuring surface tension. The principal object was to bring about economy in time of operation by elimination of the photographic process involved. This was achieved through the development of an instrument called the tangeometer.

2. By use of the tangeometer, the time required for a surface tension determination is reduced to 25 per cent of that formerly required. Economy of operation was improved still further because of the elimination of the need for the photographic materials formerly used.

3. This modified pendant drop method of measuring surface tension appears to be very promising for many other applications where there is a need for speedy surface tension determinations.

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The Effect of Chlorophyllins on Various Odorants as Measured by Surface Tension Changes of Liquids*

By J. A. CAMPBELL and J. E. CHRISTIAN

The effect of chlorophyllins on various odorants was studied using an improved surface tension method for the detection of odorant substances. It was found that certain odorants could be detected and measured by the technique while others could not and that the effect of chlorophyllins on the various odorants could be studied by the method employed.

A NEED still exists for a scientific, reliable, and speedy method of evaluating deodorants and odor preventives. Since all of the present methods of measuring odors are based upon the human olfactory system, they are consequently subjective in nature and thus open to a certain amount of criticism. The ideal basis of a method of odor measurement would be some physical or chemical phenomenon which could be related to the odor in question and thereby increase the precision over methods involving human perception.

One such phenomenon, which was shown by Tanyolac and Eaton (1) to have possibilities, was that of surface tension change of a liquid when in the presence of certain odors. This technique was further explored by Campbell, Ahmed, Christian, and Eaton (2), and an attempt was made to make a limited test of the deodorizing ability of chlorophyllins. Because of the time consuming nature of the technique, extensive tests could not be carried out.

Upon the successful development of the tangeometer (3), an opportunity was available to study the deodorizing effects of chlorophyllins more extensively. This new rapid method also made it possible to re-examine critically the surface tension technique for measuring the concentration of odorant molecules and to evaluate more clearly its capabilities as well as its limitations.

Tests were carried out using the newly developed tangeometer. The principle of the method is the same as that outlined by Campbell, *et al.* (2), and the interpretation of the results is the same. The greater the concentration of odorant present, the greater will be the lowering in surface tension produced. No change in surface tension indicated the complete absence of the odorant.

EXPERIMENTAL

Testing of Chlorophyll Derivatives for Deodorizing Ability against Certain Substances.—In the testing of chlorophyll derivatives for deodorizing ability, it was desirable to select odorants of a practical nature which could be associated with humans or human habits. For this reason, ethanol, isoamyl alcohol, butyric acid, butyl acetate, skatole, indole, diethylamine, dodecyl mercaptan, and benzyl mercaptan were selected.

The general procedure followed in testing the odorant substances with chlorophyll derivatives was as follows: a definite amount of the chlorophyll derivative was accurately weighed in a 10-ml. volumetric flask. To the flask was added, by means of a pipet, a definite volume of the odorant solution of known concentration. The concentration of the solution of the odorant depended upon the strength which was required to produce a minimum lowering in surface tension of five dynes/cm. in fifteen minutes when tested without the presence of the chlorophyll derivatives. The mixture of chlorophyll derivative and the solution of the odorant was shaken in a mechanical shaker for a period of fifteen minutes before introduction into the chamber for measurement of the presence of an odorant. Various quantities of the chlorophyll derivatives were used in an attempt to find the quantity which would eliminate entirely the odor contained in the flask.

It was found that the total deodorization could not be obtained with ethanol, isoamyl alcohol, *n*-butyl acetate, butyric acid, diethylamine, and dodecyl mercaptan, even when treated with large quantities of chlorophyllins. A reduction in the surface tension was evident in each case, but the reduction was not in proportion to the quantity of chlorophyllins added. The results are shown in Table I.

Odorants Which Could Not Be Detected by the Technique.—A number of odorants which were tested using water as the pendant drop were found not to affect the surface tension. Odorants which fall into this class are dodecyl mercaptan, skatole, indole, aniline, thiamine, coumarin, benzyl mercaptan, aqueous extract of onion, and asparagus urine.

The inability to detect certain substances using the pendant water drop demonstrated the need for additional test liquids. Therefore, an attempt was made to enlarge the number of such suitable test liquids.

In the case of tertiary dodecyl mercaptan, the problem was solved by substituting Clark and Lubs buffer solutions (6) in the place of water. It was found that 5 ml. of a saturated aqueous solution of the mercaptan would produce an average lowering of surface tension upon the buffer of 10.1 dynes/cm. in thirty minutes. The lowering was found to be independent of the *p*H of the buffer. Although the use of the above buffers permitted the detection of tertiary dodecyl mercaptan, these buffers, as well as

* Received August 28, 1954, from the School of Pharmacy, Purdue University, West Lafayette, Ind.

the use of water, were of no value in detecting skatole, indole, aniline, thiamine, coumarin, benzyl mercaptan, aqueous onion extract, and asparagus urine.

TABLE I.—THE DEODORIZING EFFECTS OF CHLOROPHYLLINS ON CERTAIN SUBSTANCES

Odorant	Deodorant (mg.)	Time (min.)	Surface Tension Lowering (dynes/cm.)
Ethanol[a]	None	30	13.3
Ethanol[a]	100[b]	30	12.6
Ethanol[a]	500[b]	30	10.7
Ethanol[a]	750[b]	30	9.2
Tertiary dodecyl mercaptan[c]	None	30	10.1
Tertiary dodecyl mercaptan[c]	100[d]	30	7.8
Tertiary dodecyl mercaptan[c]	250[d]	30	4.9
Tertiary dodecyl mercaptan[c]	500[d]	30	4.5
Isoamyl alcohol[e]	None	30	23.5
Isoamyl alcohol[e]	200[b]	30	16.0
Isoamyl alcohol[e]	500[b]	30	13.3
Isoamyl alcohol[f]	None	45	19.4
Isoamyl alcohol[f]	500[b]	45	12.8
Isoamyl alcohol[f]	750[b]	45	12.8
Butyric acid[g]	None	45	6.8
Butyric acid[g]	100[h]	45	6.6
Butyric acid[g]	500[h]	45	4.1
n-Butyl acetate[i]	None	30	16.2
n-Butyl acetate[i]	500[d]	30	12.4
n-Butyl acetate[j]	None	30	14.8
n-Butyl acetate[j]	500[d]	30	9.8
Diethylamine[k]	None	30	16.8
Diethylamine[k]	500[d]	30	14.3

[a] Five ml. of a 15% aqueous solution. [b] Na, K, Cu chlorophyllins (80%). [c] Five ml. of a saturated solution (aqueous) at 25°. [d] Na, K, Cu chlorophyllins (77.4%). [e] Five ml. of a 1% aqueous solution. [f] Two ml. of a 1% aqueous solution. [g] Five ml. of 5% aqueous solution. [h] Na, K, Cu chlorophyllins (80%) mixed with four drops of Tween®-40. [i] Five ml. of a saturated aqueous solution. [j] Two ml. of a saturated aqueous solution. [k] Five ml. of a 2% aqueous solution.

The following organic liquids which have a relatively high surface tension were screened for their applicability to the role of test liquid in the surface tension technique for measuring the above odorants: *ortho*-nitrotoluene, nitrobenzene, dimethyl sulfate, 2-nitropropane, furfural, 1,4-dioxane, *ortho*-toluidine, quinoline, *meta*-cresol, Tween®-60, Span®-80, acetic acid, and U. S. P. heavy mineral oil. None of the liquids tested showed promise in broadening the application of the method over the use of distilled water.

The Evaluation of Chlorophyll Derivatives Using Cineol and Eugenol as Odorants.—With the use of the tangeometer, it was found that 5 ml. of a saturated aqueous solution of cineol and of eugenol had no effect on the surface tension when mixed with 750 mg. and 250 mg., respectively, of Na, K, Cu chlorophyllins over a period of fifteen minutes. Two hundred fifty milligrams of Na, K, Cu chlorophyllins eliminated the surface tension effects of 0.5 ml. of a saturated solution of menthol after the same length of contact.

Cineol and eugenol were selected as the best available odorants for the use in *in vitro* evaluation of chlorophyll derivatives.

A saturated aqueous solution of cineol was prepared and maintained at 25° by placing the container in a constant temperature bath associated with the testing cell. A 250-mg. sample of the chlorophyll derivative being tested was accurately weighed in a 10-ml. flask. Exactly 5 ml. of the saturated solution of cineol was added by means of a pipet. The flask was well stoppered and shaken in a mechanical shaker for a period of one minute; after which, it was allowed to stand for an additional fourteen minutes. The mixture was introduced into the chamber for measurement. The test liquid used was distilled water maintained at 25°. The surface tension was measured at five-minute intervals for a period of fifteen minutes after introduction. Each experiment was duplicated to insure accuracy.

A total of 13 chlorophyll derivatives were tested according to the above procedure as well as six other substances not related to chlorophyll. Their relative efficiencies in reducing the concentration of cineol are shown in Table II, where the substances are listed in order of decreasing activity.

Using a saturated aqueous solution of eugenol as odorant, all of the chlorophyll derivative samples listed in Table II, except 12 and 13 which are not available, were treated in a manner identical to that with cineol. The samples are listed in Table III in order of decreasing activity.

The small amount of lowering produced by eugenol does not provide sufficient latitude for as critical evaluation as in the case with the cineol.

TABLE II.—THE SURFACE TENSION EFFECTS OF CHLOROPHYLL DERIVATIVES AND CERTAIN OTHER SUBSTANCES UPON CINEOL

Substance[a]	Surface Tension Lowering (dynes/cm.)
1. Saturated solution of cineol alone	20.4
2. Na, K, Fe chlorophyllins, 85.5% Co. A.	9.2
3. Na, K, Cu chlorophyllins, 89.9% Co. A.	10.2
4. Na, K, Cu chlorophyllins, 80.0% Co. A.	12.3
5. Na, K, Cu chlorophyllins, 77.4% Co. A.	12.7
6. Na, K, Cu chlorophyllins, 52.9% Co. A.	13.7
7. Na, K, Fe chlorophyllins, 77.2% Co. A.	14.4
8. Na, Cu chlorophyllins, 75.0% Co. B.	14.65
9. K, Fe chlorophyllins, no stated concn. Co. C.	15.2
10. Na, Cu chlorophyllins, 75.0% Co. D.	15.6
11. Na, Cu chlorophyllins, 75.0% Co. E.	15.9
12. Rhodacin, no stated concn.	16.5
13. Rhodaphyll, no stated concn.	16.75
14. Na, K, Mg chlorophyllins, 25.0% Co. A.	17.1
Other Substances Tested	
1. Activated charcoal	0.0
2. Animal charcoal	13.8
3. Sodium thiosulfate	16.2
4. Sodium benzoate	16.9
5. Hemin	18.3
6. Potassium permanganate	20.2

[a] 250 mg. was used in each case.

TABLE III.—THE SURFACE TENSION EFFECTS OF CHLOROPHYLL DERIVATIVES AND CERTAIN OTHER SUBSTANCES UPON EUGENOL

Substance*	Surface Tension Lowering (dynes/cm.)
1. Saturated solution of eugenol alone	5.6
2. Na, K, Fe chlorophyllins, 85.5% Co. A.	0.0
3. Na, K, Cu chlorophyllins, 89.9% Co. A.	0.0
4. K, Fe chlorophyllins, no stated concn. Co. C.	0.0
5. Na, Cu chlorophyllins, 75.0% Co. D.	0.0
6. Na, Cu chlorophyllins, 75.0% Co. E.	0.75
7. Na, Cu chlorophyllins, 75.0% Co. B.	1.05
8. Na, K, Cu chlorophyllins, 80.0% Co. A.	1.2
9. Na, K, Cu chlorophyllins, 77.4% Co. A.	1.35
10. Na, K, Cu chlorophyllins, 52.9% Co. A.	1.50
11. Na, K, Mg chlorophyllins, 25.0% Co. A.	2.25
Other Substances Tested	
1. Activated charcoal	0.0
2. Animal charcoal	0.0
3. Potassium permanganate	0.0
4. Sodium benzoate	2.4

* 250 mg. used in each case.

DISCUSSION

The greatest obstacle to the application of the surface tension technique to general odorant measurement is the limited number of odorants which can be detected and measured using water as the test liquid. Additional test liquids are needed, the surface tension of which is sensitive to those odorants which do not affect water.

Some indication of the requirements for test liquids can be deduced from the results of past work. It appears that for an odorant to affect the surface tension of water, for example, the odorant molecule must possess at least one "water attracting" group in its structure. Compounds which contain a hydroxyl group such as the alcohols or eugenol affect the surface tension of water readily. Cineol contains an ether linkage which is capable of bonding with water and hence lowers the surface tension of water. Esters are very effective in lowering the surface tension of water, which is probably due to the carbonyl group. Skatole and indole, on the other hand, have no effect upon the surface tension of water. This lack of action might be explained by the fact that they are ring compounds with no water attracting group exposed. In future searches for surface tension sensitive liquids, it would be well to consider the relationship between the attractive forces of the molecular structures of the compound under test and the test liquid.

The mechanism of the limited odor reduction of chlorophyll derivatives *in vitro* is not known. From observations in the work with cineol, it appears not to be one of oxidation or reduction since potassium permanganate has no effect in reducing the surface tension effects of cineol. Sodium thiosulfate likewise had very little effect, approximately the same as sodium benzoate. It appears that the most likely explanation of the effectiveness of chlorophyllins in the deodorization of cineol and eugenol is one of adsorption or formation of complexes with greatly lowered vapor pressure.

SUMMARY AND CONCLUSIONS

1. The surface tension technique was critically examined using the tangeometer; and it was found that certain odorants could be detected and measured by the technique, while other odorants could not. The odorants which could be detected were cineol, eugenol, ethanol, isoamyl alcohol, butyric acid, thioglycolic acid, butyl acetate, diethylamine, dodecyl mercaptan, and menthol. Those odorants which could not be detected were skatole, indole, aniline, thiamine coumarin, benzyl mercaptan, aqueous extract of onion, and asparagus urine.

2. Only partial odor reduction of ethanol, isoamyl alcohol, butyric acid, *n*-butyl acetate, dodecyl mercaptan, and diethylamine could be obtained by the use of chlorophyllins.

3. A number of liquids were screened for their applicability to the role of test liquid, along with water and buffer solutions. No additional liquids were found which showed promise as test liquids.

4. Cineol and eugenol, being among the only three odorants which were completely deodorized by chlorophyllins and which could be detected by the device, were used to evaluate chlorophyll derivatives for deodorizing efficiency.

5. Thirteen chlorophyll derivatives, representing five manufacturers, were evaluated against five milliliters of a saturated solution of cineol. The effects of six nonchlorophyll compounds were determined as controls.

6. Eleven chlorophyll derivatives as well as four nonchlorophyll compounds were evaluated against five milliliters of a saturated solution of eugenol.

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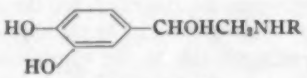
The Analysis of Solutions of Epinephrine and Norepinephrine*

By LLEWELLYN H. WELSH

A method of analysis is described which is applicable to solutions of epinephrine and norepinephrine, such as epinephrine injection U. S. P. and levarterenol bitartrate injection N. N. R. The method depends on the quantitative conversion of the substances into O^3,O^4, N-triacetyl derivatives which may be extracted into chloroform, weighed, identified, and examined polarimetrically for the determination of stereochemical composition. Excellent recoveries of the physiologically potent *l*-isomer are obtained, and results of analyses of solutions of epinephrine show very good agreement with potencies determined by the U. S. P. XIV dog bioassay. By supplementing the method with partition chromatography of the acetylation product, the norepinephrine in a solution of U. S. P. epinephrine may be quantitatively isolated as its triacetyl derivative and determined colorimetrically after deacetylation and oxidation to noradrenochrome.

SINCE 1926, when epinephrine (I) and a solution of its hydrochloride were first recognized by the United States Pharmacopeia, the dog bioassay has been the method prescribed by that compendium for estimating the potency of solutions of the drug. This assay (1) measures the increase in arterial pressure caused by intravenous injection of the solution into the test animal, compares this effect with that produced by a standard solution of epinephrine, and expresses the result in terms of *l*-epinephrine per unit volume of solution.



I, R = CH_3
II, R = H

Several colorimetric or fluorometric methods have been developed for assaying solutions of epinephrine (2). Despite the relative simplicity of these methods and the inconvenience of the bioassay, the latter has been maintained as the official method principally because of the fact that, although excellent agreement may be demonstrated between such methods and the bioassay when freshly prepared solutions are used in the comparison, the former methods frequently give considerably higher results than does the biological method in the assay of solutions which have aged or been subjected to a certain intensity of heat during sterilization. This discrepancy may be ascribed to the circumstance that loss of potency is largely due to partial conversion of *l*-epinephrine to its *d*-isomer, which has about 5 per cent of the biological activity of the *l*-form (3), and that the enantiomorphic forms of a substance give precisely the same color reactions. The results of Hald (4) definitely relate racemization to the loss of potency accompanying the ageing of 10 per cent spray solutions. Racemization as a source of deterioration is also strongly suggested by correlation of the facts that optically active epinephrine racemizes with relative ease in the presence of aqueous acid (5, 6) and, as shown by Berry and West (7), that the biological potency of a 0.1 per cent solution of epinephrine undergoes a loss, not ascribable to oxidation, which increases in rate as the *p*H decreases from a value of 4.2.

Rosenblum, Goldman, and Feldman (8) appear to have been the first to describe a chemical method designed to measure the quantity of potent *l*-isomer in dilute solutions of the drug. In examining a 1 per cent solution by their method, the optical rotation of the solution is determined, and from this value is calculated the amount of *l*-isomer in excess of the portion, the rotatory effect of which is nullified by the contribution of the *d*-isomer present. The solution is then assayed colorimetrically for its content of both isomers by Doty's method (2), and the total *l*-epinephrine is calculated as the arithmetic mean of this value and that determined polarimetrically. In assaying a 0.1 per cent solution, such as epinephrine injection U. S. P., the solution is concentrated to one-tenth of its volume by vacuum distillation in order to obtain a measurable optical rotation. The authors report re-

* Received July 2, 1955 from the Department of Health, Education and Welfare, Food and Drug Administration, Division of Pharmaceutical Chemistry, Washington, D. C.

The author wishes to acknowledge his indebtedness to the following: F. A. Maurina, director, analytical laboratories, Parke, Davis and Company, for supplying the three solutions of epinephrine hydrochloride and data concerning their composition and activity in bioassays; Roland H. Noel, executive director of control, Bristol Laboratories, Inc., for furnishing the aged commercial solution of epinephrine and data on its biological potency; Irwin S. Shupe, assistant director of control, Winthrop-Stearns, Inc., for donating generous samples of *dl*-epinephrine base, *l*-norepinephrine bitartrate, and *dl*-norepinephrine hydrochloride; Albert H. Tillson, Division of Microbiology, Food and Drug Administration, for determining crystallographic properties; Paul J. Vollmer, Division of Cosmetics, Food and Drug Administration, for determining infrared absorption spectra.

sults which deviate from biologically determined values within the range of 1 to 13 per cent. They propose the method as a simple and inexpensive one by means of which a manufacturer of pharmaceuticals may control his product and determine its shelf life.

The method of Hellberg (9) is fundamentally the same as that of Rosenblum, Goldman, and Feldman. Concentration of a 0.1 per cent solution is effected conveniently by the use of an ion exchange resin instead of by the troublesome process of vacuum distillation.

Inherent in the methods of Rosenblum, Goldman, and Feldman (8) and Hellberg (9) are the assumptions that no chromogenic substances other than epinephrine are present, and that the rotatory power of the solution is due solely to *l*-epinephrine. Because of this, the methods are scarcely suitable for use in situations, e. g., in regulatory work, in which the analyst has no prior knowledge of the nature of the materials used in compounding the sample at hand. Under such conditions, in order that a chemical method may be relied upon to give an accurate measure of the potency of a solution in terms of the official drug, it must permit establishment of the fact that the active ingredient is epinephrine and provide for an accurate determination of its stereochemical composition. These requirements practically restrict the method to one which is based on the quantitative isolation of epinephrine, as such or in the form of a suitable derivative, so that it may be examined by the methods of qualitative organic analysis and polarimetry. It is the purpose of this paper to report a method of analysis of this type.

In dilute aqueous solution, epinephrine is quantitatively converted into its O^3, O^4, N-triacetyl derivative (10). Unlike the parent base, the derivative is relatively stable, and may be easily extracted into chloroform.[1] After removal of solvent, the residue is weighed and its specific rotation determined in chloroformic solution. From these data is calculated the quantity of *l*-epinephrine in the sample taken for analysis. In Table I are listed results of the application of the procedure to solutions containing epinephrine of different stereochemical compositions (Solutions 1, 2, 3).

The derivative recovered from the solution used in the determination of optical rotation may be identified by its melting point, ultraviolet and infrared absorption spectra, and optical crystallographic properties. The pure *l*-derivative has a melting point of 94–95° and $[\alpha]_D$ −94.7° (10). If the derivative, reflecting the stereochemical composition of the parent epinephrine, is not of high optical purity it will melt over a rather wide range, since it consists of a mixture of triacetyl-*l*-epinephrine and triacetyl-*dl*-epinephrine, m. p. 104–105° (11), and thus behaves as though a foreign substance were present in the original solution. This difficulty is eliminated by heating the derivative with dilute acid, which deacetylates and completely racemizes it regardless of its stereochemical composition. The racemic triacetyl derivative obtained after acetylating the resulting solution of *dl*-epinephrine melts over a relatively narrow range.

l-Norepinephrine (*l*-arterenol, II), a vasopressor like epinephrine, is listed in N. N. R. (12) as the crystalline bitartrate (levarterenol bitartrate) and as a solution of this salt 0.1 per cent with respect to norepinephrine base. As in the case of epinephrine, the *l*-isomer is physiologically many times more active than the *d*-isomer (3). In aqueous solution norepinephrine acetylates in exactly the same manner as does epinephrine, and for this reason the amount of *l*-isomer in a solution of the bitartrate may be determined by the same procedure described for the assay of solutions of epinephrine. The calculations differ from those applicable to the epinephrine assay with respect to the values of the rotatory and gravimetric factors. Results of assays of a solution of norepinephrine are listed in Table I (Solution 4).

The derivative recovered from the solution used for the determination of specific rotation in the assay is available for qualitative tests. The purest triacetyl-*l*-norepinephrine prepared in this laboratory had a melting point of 69.5–71°, $[\alpha]_D$ −81°. The racemic derivative melted at 116.5–117.5°.

l-Norepinephrine has been employed as a therapeutic agent for but a relatively short time, and apparently no data have accumulated regarding its susceptibility, as the bitartrate, to racemization in aqueous solution. Norepinephrine is, however, closely related to epinephrine in structure, and in aqueous solution, as the hydrochloride, has been reported to suffer what is evidently a stereochemical change when exposed to temperatures above 50° for but a few minutes (13). In view of these considerations, it would appear that a method of analysis such as the above, which will provide a measure of the *l*-isomer, may have certain advantages over a

[1] The relative insolubility of epinephrine, norepinephrine, and the related substances, phenylephrine and synephrine, in chloroform and similar solvents appears to be related in some way to the presence of basic and acidic (phenolic) groups in the same molecule.

spectrophotometric method, such as that described in N. N. R. (14), which is devoid of stereospecificity.

In 1949 it was recognized that *l*-norepinephrine is usually present in U. S. P. epinephrine obtained from natural sources, and occasionally constitutes approximately 20 per cent of the official drug (15). The present method will permit an accurate measurement of the U. S. P. epinephrine, i. e., *l*-epinephrine plus *l*-norepinephrine, in a solution even though one employs in the calculations rotatory and gravimetric factors which are theoretically applicable only when epinephrine is the sole molecular species present. This is due to the circumstance that these factors do not differ too greatly from those applicable to the determination of norepinephrine, and the error due to the difference in rotatory factors is partly compensated by that due to the difference in gravimetric factors. It may be calculated that the net error is less than 1 per cent when the U. S. P. epinephrine contains as much as 20 per cent of the nor-compound. In Table I are listed the results of assays of solutions in which the U. S. P. epinephrine contained from 2 to 20 per cent of norepinephrine (Solutions 5, 6, 7).

The acetylation product from a solution of U. S. P. epinephrine containing norepinephrine is not suitable for a complete qualitative examination until it has been separated into its component triacetyl derivatives. This may be accomplished by partition chromatography, in which water supported on Celite® functions as the stationary phase, and benzene as the mobile phase. Since the chromatographic separation is a quantitative one, the fraction of triacetylnorepinephrine may be employed in determining the total (*d* plus *l*) norepinephrine in the original solution. For this purpose the separated derivative is deacetylated with hot dilute hydrochloric acid, and the regenerated norepinephrine is estimated colorimetrically after oxidation to noradrenochrome by the procedure of von Euler and Hamberg (16). Results of determinations of norepinephrine in solutions containing epinephrine and norepinephrine are presented in Table I (Solutions 5, 6, 7).

The relatively small quantity of norepinephrine present in a 0.1 per cent solution of U. S. P. epinephrine renders impracticable a determination of the stereochemical composition of the substance by polarimetric examination of its separated triacetyl derivative. The close structural relationship of epinephrine and norepinephrine would appear to make reasonable the assumption that they racemize at comparable rates. If the assumption is valid, the specific rotation of the mixture of derivatives will provide a measure of the stereochemical composition of the norepinephrine, as well as of the epinephrine, in a solution of natural epinephrine.

TABLE I.—RESULTS OF ANALYSES OF AQUEOUS SOLUTIONS OF EPINEPHRINE (E) AND NOREPINEPHRINE (N)

Solution	Present, mg./100 ml.	Found, mg./100 ml.	Recovery, %
1	107.0 *l*-E	107.7, 107.8 U. S. P. E[a]	100.7, 100.8
2	90.0 *l*-E 10.0 *d*-E	90.0, 90.0 U. S. P. E	100.0, 100.0
3	50.0 *l*-E 50.0 *d*-E	50.8, 50.4 U. S. P. E	101.6, 100.8
4	100.4 *l*-N	100.7, 100.6 *l*-N	100.3, 100.2
5	98.0 *l*-E	99.9, 100.3 U. S. P. E	99.7, 100.1
	2.20 *l*-N	2.06, 2.11 N[b]	94, 96
6	90.0 *l*-E	98.7, 99.1 U. S. P. E	98.7, 99.1
	10.0 *l*-N	9.5, 9.5 N	95, 95
7	60.0 *l*-E	75.1, 74.4, 74.7 U. S. P. E	100.1, 99.2, 99.6
	15.0 *l*-N 20.0 *d*-E		
	5.0 *d*-N	19.1, 18.6, 19.1 N	95, 93, 95

[a] The quantity of U.S.P. epinephrine is equivalent to the sum of the quantities of the levorotatory isomers of epinephrine and norepinephrine, i. e., *l*-E + *l*-N.
[b] The letter, N, without definitive prefix, symbolizes total norepinephrine, i. e., *l*-N + *d*-N.

EXPERIMENTAL

Melting Points Are Corrected.

Preparation of Solutions Analyzed.—With the exception of Solution 1, suitable quantities of the following salts were employed in preparing the solutions listed in Table I: synthetic *l*-epinephrine bitartrate (Winthrop-Stearns) twice recrystallized from ethanol-water; synthetic *l*-norepinephrine bitartrate monohydrate, purified as described in a subsequent section; *dl*-epinephrine oxalate; and *dl*-norepinephrine hydrochloride. The solutions, which also contained 0.5% chlorobutanol and 0.1% sodium metabisulfite, were analyzed within twenty-four hours after their preparation.

Solution 1 was prepared by Parke, Davis and Company for investigational use. It contained natural *l*-epinephrine (free of nor-compound) as the hydrochloride, and was preserved with chlorobutanol and bisulfite.

Determination of U. S. P. Epinephrine.—Exactly 30 ml. of solution was transferred to a 125-ml. separatory funnel having a tightly-fitting stopper and stopcock, and chlorobutanol was removed by extracting the solution three times with 25-ml. portions of reagent grade carbon tetrachloride. Each shakeout was conducted for one minute, and after each the solvent, including the large drop which usually floats on the surface of the water until dislodged, was separated as completely as possible.

The stopper and mouth of the funnel were rinsed with a few drops of water, and the rinsings were combined with the solution in the funnel. Four drops of starch T. S. were added, and bisulfite was destroyed by adding iodine and potassium iodide T. S.,

dropwise, while the funnel was continuously swirled, until a blue color persisted. The blue was immediately discharged by the dropwise addition of 0.1 *N* sodium thiosulfate. Two and one-tenth grams of sodium bicarbonate was added (contact of the salt with the wet mouth of the funnel was avoided), and the funnel was swirled a few seconds to dissolve most of the bicarbonate.

By means of a calibrated 1-ml. hypodermic syringe (without needle) exactly 1 ml. of A. C. S. reagent grade acetic anhydride was rapidly injected into the contents of the funnel, and the funnel was immediately stoppered securely and shaken vigorously until the evolution of carbon dioxide had ceased (about seven minutes). Pressure in the system was released when necessary by momentarily inverting the funnel and cautiously opening the stopcock. The mixture was allowed to stand five minutes, then was extracted with six 30-ml. portions of U. S. P. chloroform. Each extract was filtered through a chloroform-washed compact pledget of absorbent cotton into a beaker, and the combined extracts were evaporated to a small volume or to dryness on the steam bath in a current of air. By means of small portions of chloroform the residue was transferred to a tared 50-ml. beaker, and evaporation was continued until the solvent had been removed. The beaker and contents were heated at 105° for thirty minutes, allowed to cool in a desiccator, and the weight of the amorphous residue of acetylation product was determined.

Exactly 5 ml. of U. S. P. chloroform was added to the beaker, which was immediately covered with a watch glass and swirled gently until the residue had completely dissolved. The optical rotation of the solution was determined in a 200-mm. semimicro polariscope tube, and the specific rotation of the acetylation product was calculated. The quantity of U. S. P. epinephrine, E, in the sample analyzed was calculated by means of the easily-derived equation:

$$E = (0.5 + 0.5\,R/93) \times W \times 0.5923$$

in which *W* is the weight of the acetylation product, *R* its specific rotation, 0.5923 a gravimetric conversion factor, and 93 a rotatory factor.

In the analysis of solutions of norepinephrine, the calculations employed were the same, except that values of 0.5730 and 80 were substituted, respectively, for the gravimetric and rotatory factors.

Separation of Triacetylepinephrine and Triacetylnorepinephrine.—*Chromatographic Tube.*—This was prepared from a 200 x 25-mm. test tube. In it was placed a wad of glass wool (#800 Pyrex catalog) which was compressed tightly at the juncture of the tube and its stem by means of a packing rod, prepared by flattening the end of a glass rod, about 300 mm. in length, to a circular head about 21 mm. in diameter.

Chromatographic Column.—In a 250-ml. beaker were placed 10 Gm. of #545 Celite® and 175 ml. of benzene.[2] While the mixture was stirred vigorously and continuously, 7.0 ml. of water was added, drop by drop, so as to produce a uniform solid phase. About one-tenth of the solid phase, under benzene, was transferred to the chromatographic tube and compressed firmly and evenly with the packing rod. While a column of benzene was maintained in the tube, the remainder of the solid was added in 5 approximately equal portions. Each portion was compressed firmly and evenly before adding the next. Solid phase adhering to the tube above the finished column was removed by swabbing with absorbent cotton affixed to a stiff wire. Columns prepared in this manner were 65–70 mm. in height and permitted a flow of *ca.* 2.5 ml. of benzene per minute under a head of 90 mm. of solvent. A layer of benzene was maintained above the column until it was time for its use.

Procedure.—The solution of acetylation product in the polariscope tube was combined with the unused chloroform solution, which had been preserved in a small, tightly-closed vessel during the determination of specific rotation. A 4-ml. aliquot was pipetted into a 50-ml. Erlenmeyer flask, and solvent was completely removed by evaporation on the steam bath in a current of air. The residue was completely dissolved in exactly 5 ml. of dry benzene by swirling and gentle warming.

The supernatant layer of benzene was removed from the chromatographic tube by aspiration, a 4-ml. aliquot of the cooled benzene solution of acetylation product was pipetted onto the top of the column, and a graduated cylinder was immediately placed under the tube for the collection of effluent. As soon as the last of the solution had been absorbed by the column, the wall of the tube was rinsed down three times with 2-ml. portions of benzene. The rinse was allowed each time to be completely absorbed by the column before adding the next portion. When the last of the rinsings had been absorbed, benzene was poured carefully into the tube to a height of 80–90 mm. above the top of the column, and was maintained at this level until a total of 160 ml. of effluent had been collected. The tip of the stem was rinsed thoroughly with chloroform, and the combined rinsings and effluent were reserved for the isolation of triacetylepinephrine.

The layer of benzene above the column was removed by aspiration, a clean receiver was placed under the tube, and U. S. P. chloroform was allowed to pass through the column until 100 ml. of effluent, containing triacetylnorepinephrine, had been collected. The effluent was evaporated to dryness, ultimately in a 50-ml. beaker, in such a way as to confine the residue near the bottom of the vessel.

Colorimetric Determination of Norepinephrine.—To the beaker containing triacetylnorepinephrine was added an accurately-measured volume (5 or 10 ml.) of exactly 0.5 *N* hydrochloric acid, the system was warmed gently, and the residue was dissolved by stirring and rubbing with a rubber policeman. The solution was poured into a 150 x 15-mm. test tube which was then placed in a boiling water bath and closed but loosely with its glass stopper in order to permit the escape of expanding air. After five minutes the tube was tightly stoppered, and was maintained at 100° for thirty additional minutes. The tube was removed from the bath and cooled to room temperature while the stopper was loosened sufficiently to prevent the formation of a vacuum. The contents of the tube were mixed

[2] A. C. S. reagent grade benzene was distilled in an all-glass apparatus. The distillate was shaken with water a few minutes, separated, and filtered through filter paper. Unless otherwise specified, this water-saturated solvent was used in the chromatographic work.

thoroughly, and an accurately-measured aliquot (0.5–1 ml.) was transferred to another glass-stoppered test tube. The acid was neutralized by the addition of the calculated amount of sodium bicarbonate (42 mg./ml.), and, after effervescence had ceased, the volume was brought up to exactly 2.5 ml. with the necessary quantity of water. Exactly 2.5 ml. of concentrated *p*H 6 buffer (prepared with half the volume of water specified by the formula) was added, and the solution was mixed by swirling. Four drops of 0.1 *N* iodine were then added and the solution was swirled. Exactly three minutes (timed by a stopwatch) after the addition of iodine, 6 drops of 0.1 *N* sodium thiosulfate were added, the tube was stoppered and its contents were thoroughly mixed. By means of a Beckman DU spectrophotometer (1-cm. cell, *p*H 6 buffer blank) the absorbance of the solution at 520 mμ was measured three minutes (±30 seconds) after the addition of thiosulfate. The absorbance was compared with a standard curve, and the quantity of norepinephrine in the original solution was calculated.

The standard curve was based on data obtained by the following procedure.

A standard solution of norepinephrine was prepared by dissolving 19.9 mg. of *l*-norepinephrine bitartrate monohydrate in 100 ml. of *p*H 6 buffer. Accurately-measured aliquots of standard soiution, each ml. of which represented 0.100 mg. of norepinephrine base, were transferred to 4 glass-stoppered test tubes to provide a series in which the respective tubes contained 0.5, 1.0, 2.0, and 2.5 ml. of solution. After addition of sufficient *p*H 6 buffer to each tube to make a final volume of exactly 5 ml., color was developed and its intensity measured as described above. The best straight line based on the plotted absorbances (range, *ca.* 0.14–0.70) followed Beer's law. The maximum deviation of the plotted absorbances from the line was 1%.

O³,O⁴, N-Triacetyl-*l*-norepinephrine.[1]—Commercial *l*-norepinephrine bitartrate monohydrate was dissolved in an equal weight of warm (45–50°) water, a volume of 95% ethanol equal to 10 times that of the water was added, the system was seeded, and crystallization was allowed to proceed until essentially complete at room temperature. Crystallization was completed at *ca.* −10°, and the solid was filtered off, washed with cold 95% ethanol, then with ether, and was air-dried. Recovery was 90%. Preliminary experiments showed that after 6 such purifications, additional recrystallizations did not increase the rotatory power of amorphous triacetyl derivative obtainable from the bitartrate. A batch of commercial bitartrate yielded a derivative of $[\alpha]_D^{25}$ −76° (1% in U. S. P. $CHCl_3$). After 3, 6, and 9 recrystallizations of the salt the specific rotation of amorphous derivative from the respective crops was −78, −81, and −80°.

Two grams of bitartrate (6× recrystallized) was dissolved in 100 ml. of water in a 500-ml. separatory funnel, 11.5 Gm. of sodium bicarbonate was added, and acetylation was effected with 5 ml. of acetic anhydride in the manner described in the assay procedure, except that the anhydride was added in portions of 2, 2, and 1 ml. After the reaction was complete, the derivative was extracted with six 50-ml. portions of chloroform. After removal of solvent from the filtered extracts, the resinous derivative was induced to crystallize by triturating it with 30–60° petroleum ether in the presence of seed. Crystallization was slow, requiring about two hours for completion. The yield of white powder was 1.66 Gm. (95%): $[\alpha]_D^{25}$ −79.7°.

One and forty-six one-hundredths grams of the crystalline powder was dissolved in 2.95 ml. of acetone, and to the stirred solution was added a volume of toluene (*ca.* 17 ml.) slightly less than that which would produce a permanent turbidity. The solution was filtered and seeded with *ca.* 5 mg. of previously recrystallized material. The system was stirred frequently at room temperature for three hours, and then was allowed to stand for sixteen hours at the same temperature. It was then kept at 10° for three hours, and was finally maintained at −10° for two hours. Attempts to shorten the time of recrystallization by rapid cooling or by diluting the system with more toluene caused the derivative to deposit as an oil. The crystals were filtered off, washed with cold acetone-toluene, then with toluene, and finally with 30–60° petroleum ether. The recrystallized material was left overnight in a vacuum desiccator over silica gel, then was heated at 56° for two hours in a high vacuum: recovery, 1.07 Gm. (73%).

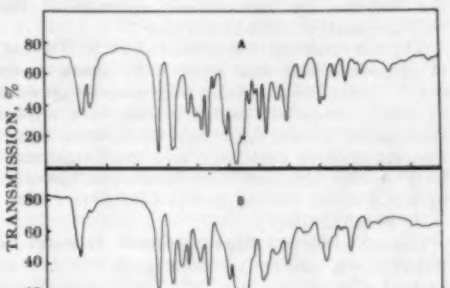


Fig. 1.—Infrared absorption spectra of crystalline O³, O⁴, N-triacetyl derivatives in 0.5-in. potassium bromide disks, recorded with a model 21 Perkin-Elmer spectrophotomer: A, triacetyl-*l*-epinephrine (1 mg. + 200 mg. KBr); B, triacetyl-*l*-norepinephrine (0.75 + 200 mg. KBr).

Anal.—Calcd. for $C_{14}H_{17}NO_6$: N, 4.74. Found: N, 4.98, 4.98. The substance exhibited the following optical crystallographic properties: *habit*, prisms with brilliant interference colors; *extinction*, parallel; *elongation*, negative; *refractive indices*, α (lengthwise) = 1.539, β = 1.548, γ (crosswise) = 1.625 (all indices ± 0.003); *optic sign*, positive; $2V$, small. It had $[\alpha]_D^{25}$ −81.3° (1% in U. S. P. $CHCl_3$). In a capillary tube, it sintered at 67.5° and melted at 69.5–71° to a clear, colorless, viscous oil in which were occluded numerous bubbles of air. The ultraviolet absorption spectrum of the substance in ethanolic solution resembles very closely that of

[1] First induced to crystallize in the control laboratory of Winthrop-Stearns, Inc.

triacetylepinephrine (10), showing maxima at 265 and 271 mμ, and minima at 247 and 268 mμ; ϵ_{265} = 550, ϵ_{271} = 495. Its infrared absorption spectrum and that of the epinephrine derivative are shown in Fig. 1.

O³,O⁴,N-Triacetyl-*dl*-norepinephrine.[4]—The racemic derivative was prepared from *dl*-norepinephrine hydrochloride by the same procedure used on the *l*-bitartrate. The product was recrystallized by dissolving in acetone (1.5 ml./Gm.) and adding 5 volumes of ether: recovery, 85%.

Anal.—Calcd. for $C_{14}H_{17}NO_6$: N, 4.74. Found: N, 4.74, 4.82.

It melted at 116.5–117.5° and had the following optical crystallographic properties: *habit*, bladed plates with brilliant interference colors; *extinction*, parallel and inclined; *refractive indices*, α = 1.479, β = 1.556, γ = 1.601 (all ± 0.003); *optic sign*, negative; 2*V*, large.

O³,O⁴, N-Triacetyl-*dl*-epinephrine.—This compound is formed, along with other products, by the interaction of acetyl chloride and *dl*-epinephrine base (11). It was prepared in this laboratory by applying to the *dl*-base or its oxalate the acetylation procedure used on the salts of norepinephrine. The product was recrystallized by dissolving it in warm acetone (1 ml./Gm.), adding 10 volumes of ether, seeding, and maintaining the system at room temperature for several hours while it was frequently stirred. Crystallization was then completed at *ca.* −10°: recovery, 85%. The rate of crystallization of the substance is much slower than that of the *l*-isomer, but substantially more rapid than that of triacetyl-*l*-norepinephrine.

The recrystallized derivative had m. p. 104–105°, in agreement with that reported by Bretschneider (11). Under the polarizing microscope it appeared as platy, irregularly-shaped prisms with brilliant polarization colors; optic axis interference figures, showing positive optic sign and large axial angle, were readily obtained with convergent polarized light; *refractive indices*, α = 1.476, β = 1.535, γ = 1.640 (all ± 0.003).

Deacetylation and Regeneration of Triacetyl Derivatives.—A solution of 52.6 mg. of O³,O⁴, N-triacetyl-*l*-epinephrine, $[\alpha]_D$ −95°, in exactly 5 ml. of 0.5 *N* hydrochloric acid was heated at 100° for thirty minutes, cooled, and transferred quantitatively to a 125-ml. separatory funnel with 25 ml. of water. The solution was extracted twice with 25-ml. portions of chloroform, and the extracts were discarded. After the addition of 2.31 Gm. of sodium bicarbonate, acetylation was effected with 1 ml. of acetic anhydride as described in the assay procedure. The O³,O⁴, N-triacetyl-*dl*-epinephrine isolated from the chloroform extracts weighed 53.6 mg. (101.9%); m. p. 101–104°, $[\alpha]_D$ −0.5°.

Application of the same procedure to O³,O⁴, N-triacetyl-*l*-norepinephrine yielded the racemic triacetyl derivative, m. p. 112–116°, $[\alpha]_D$ + 0.3°.

The specific rotations calculated for the racemic derivatives may be apparent rather than real, since they correspond to polariscopic measurements in the range of 0.005–0.010 angular degrees (200-mm. tube).

[4] First prepared in the analytical laboratory of Parke, Davis and Company.

DISCUSSION

Results of Assays.—*Determination of Total l-Catecholamines (l-Epinephrine plus l-Norepinephrine).*—Results in Table I show that the present method is capable of providing an accurate measure of total levorotatory catecholamines in 0.1% solutions of epinephrine only, of norepinephrine only, or of mixtures of the two in which the nor-compound comprises up to 20% of the total content of drugs (Solutions 1–7). Recoveries are in the range of 98.7–101.6%, and average 100.1%.

Bioassays were not run on the solutions, so it is not possible to compare these results with biologically determined values. However, a comparison is possible with respect to results obtained on three solutions which were prepared for use in a collaborative study by the Epinephrine Subcommittee of the Combined Contact Committee of the American Drug Manufacturers Association and American Pharmaceutical Manufacturers' Association. Solution A contained, per 100 ml., 107 mg. of *l*-epinephrine; Solution B, 87 mg. *l*- and 9 mg. *d*-epinephrine; Solution C, 106 mg. *l*-epinephrine and 2 mg. *l*-norepinephrine (all substances present as hydrochlorides). In the following sequence are listed mg. of U. S. P. epinephrine per 100 ml. as determined by the present method and (in parentheses) by the U. S. P. XIV bioassay: A, 107.7, 107.8 (104.2 ± 2.70, 107.9 ± 2.54); B, 88.3, 88.3 (88.1 ± 1.52, 91.4 ± 3.51); C, 106.2, 106.6 (108.4 ± 3.27, 112.7 ± 3.05).

The value of the method in providing not only a measure of biological potency but also information regarding the composition of a solution is illustrated by the results of its application to an aged commercial preparation. The exact age of this purported 1% solution of epinephrine could not be ascertained, but it was known to be several years old. The acetylation products obtained in duplicate assays by the present method corresponded, in weight, to 80.3% and 79.3% of that expected from a 1% solution, and showed $[\alpha]_D^{25}$ −54.3° and −53.9°, respectively. The derivative exhibited infrared and ultraviolet absorption spectra which were identical with those of triacetylepinephrine, and when examined crystallographically was found to consist of a mixture of triacetyl-*l*-epinephrine and triacetyl-*dl*-epinephrine. Chromatographic treatment gave a triacetylnorepinephrine fraction corresponding to but 0.3% of total catecholamines. The triacetylepinephrine fraction after deacetylation and regeneration yielded triacetyl-*dl*-epinephrine of m. p. 100–103.5°.

The duplicate quantitative data correspond to findings of 0.636 Gm. *l*-plus 0.167 Gm. *d*-epinephrine/100 ml. and 0.626 Gm. *l*-plus 0.167 Gm. *d*-epinephrine/100 ml. On the basis that the *d*-isomer has 5% of the pressor activity of the *l*-, and that the pressor effects of the isomers are additive (3), the findings correspond, respectively, to pressor potencies equivalent to 0.644 and 0.634 Gm. *l*-epinephrine per 100 ml. The combined results of 16 U. S. P. XIV bioassays run in 9 laboratories showed a potency equivalent to 0.6556 ± 0.01366 Gm. *l*-epinephrine/100 ml.

According to a colorimetric assay by Doty's method (2) the solution contained 1.05 Gm. epinephrine/100 ml., a figure some 25% higher than the

ca. 0.80-Gm. total catecholamines/100 ml. shown to be present by the weight of the triacetyl derivative. This discrepancy may be attributed to the presence of catechol derivatives other than epinephrine or norepinephrine, since the development of color depends on the presence of phenolic hydroxyl groups on adjacent carbon atoms. The nature of these chromogenic substances was not further investigated, but it is evident that their acetylation products, unlike those of the catecholamines, were hydrophilic in nature.

The solution was clear but of a brown color, the depth of which prevented a direct measurement of optical rotation in a 200-mm. tube. Fifteen milliliters was swirled five minutes with 50 mg. of Darco® G60 and was filtered. After repeating the process, the filtrate had a very pale straw color and thus satisfied the U. S. P. XIV color requirement for solutions of epinephrine. Colorimetric assay of the decolorized solution showed the equivalent of 1.02 Gm. epinephrine/100 ml., while the weight of the acetylation product corresponded to 0.769 Gm. epinephrine/100 ml. From these results it is obvious that the charcoaling affected the essential composition of the original liquid but slightly, and that the high value of the colorimetric assay could not be related to the chromogenicity of the brown pigment. The decolorized solution exhibited an optical rotation of −0.51° in a 200-mm. tube. Thus, according to the method of assay of Rosenblum, Goldman, and Feldman (8) its biological potency would be equivalent to 0.76 Gm. of U. S. P. epinephrine/100 ml.

Determination of Total Norepinephrine in the Presence of Epinephrine.—Recoveries of total norepinephrine range from 93 to 96% (Solutions 5–7, Table I) and average 94.7%. Although the results are not as close to the theoretical as are those of the determinations of total catecholamines, the accuracy of the procedure would seem to be sufficient for the purpose of establishing whether or not a solution was prepared with epinephrine satisfying essentially the requirements of an official compendium with respect to limitations on the content of nor-compound.[5]

The cause of the consistently slightly low recoveries was not investigated. It is possible that they are due to a slight amount of hydrolysis of the acetylated phenolic hydroxyl groups during the chromatographic process, since acetates of phenols frequently are hydrolyzed with relative ease, and the phenolic products would be expected to be more difficultly extractible into chloroform than would the esters.

Acetylation Reaction.—The presence of large amounts of carbon tetrachloride in the system during acetylation will interfere with the determination by retarding hydrolysis of excess anhydride, and give rise to a derivative contaminated with the reagent. The amount of solvent present after a close separation of phases causes no difficulty.

Bisulfite must be destroyed, since its presence interferes with acetylations in the presence of aqueous bicarbonate by accelerating catalytically the hydrolysis of acetic anhydride (19, 20).

The amount of bicarbonate specified is about 15% in excess of that necessary to combine with the acetic acid formed by decomposition of the anhydride. The excess should be substantially more than sufficient to neutralize the original acidity of a solution the *p*H of which falls within the limits delineated by the U. S. P. A very large excess of bicarbonate may decrease the yield of acetylation product (20).

Specific Rotations.—Although pure, recrystallized triacetyl-*l*-epinephrine has a specific rotation of *ca.* −95° in 1% chloroformic solution (10), residues obtained in the assay of purified synthetic *l*-epinephrine bitartrate gave a value of −93°. For this reason a rotatory constant of 93 is used in the calculations when epinephrine solutions are assayed. In the calculations applicable to solutions of norepinephrine, a constant of 80 instead of 81 is used for a similar reason.

The specific rotation of triacetyl-*l*-epinephrine, and probably of the corresponding norepinephrine derivative, varies somewhat with the concentration. For example, in chloroform, the value is −97° for a 0.5% solution and −91° for a 2% solution. Between 20 and 25°, for a given concentration, the specific rotation does not vary significantly.

Chromatographic Separation.—In preliminary studies on mixtures of triacetyl derivatives, the benzene effluent was collected in 20-ml. fractions. It was found that some epinephrine derivative appears in the first fraction, most of it in fractions 2, 3, and 4, a little in 5, and very little in 6. Fractions 7, 8, and 9 combined gave evidence of containing no more than a trace of derivative, as did the combined fractions 10 and 11. These observations make it evident that the separation of derivatives is a wide one, amounting to 100 ml. or more in terms of benzene effluent.

Colorimetric Measurements.—Absorption curves of the color developed in the procedure showed a broad peak at 517–523 mμ. For this reason, measurements of absorbances were made at 520 mμ instead of at 529 mμ as specified by von Euler and Hamberg (16).

Preliminary studies on the stability of the color indicated that the intensity two minutes after addition of thiosulfate was, on the average, 2% less than after one and one-half minutes. During the interval between two minutes and three and one-half minutes after the addition of thiosulfate, the intensity decreased an average of 1%. Five minutes after the addition of thiosulfate, the intensity had diminished a total of 5% from the reading taken after one and one-half minutes. By reading absorbances three minutes ±0.5 minute after adding thiosulfate, undue haste is not required, and errors due to fading of color are slight.

Qualitative Tests.—The characteristic properties of the acetylation product will be altered if the original solution contains a significant amount of a foreign substance which forms a chloroform-soluble derivative.

The U. S. P. limits of potency for solutions of epinephrine implicitly tolerate a preparation containing epinephrine of the composition 78% *l*-, 22% *d*-isomer. Such a composition would be present in a solution, originally containing 115% of the

[5] West (17) has proposed that pharmacopeial epinephrine should contain not more than 10% norepinephrine. The Fifteenth Revision of the U. S. P. (18) limits the content of nor-compound to not more than 4%.

labeled quantity of *l*-epinephrine, the potency of which had decreased to 90% as a result of partial racemization. Solutions in which extensive racemization has occurred will yield a derivative which, in solution, exhibits absorption spectra agreeing with those of the optically pure substance. The presence of racemic material may, however, be demonstrated crystallographically.

Application of Method to Various Dosage Forms.—The present scheme of analysis is applicable, after proper preliminary treatment of the sample, to all U. S. P. preparations containing epinephrine, with the exception of procaine hydrochloride and epinephrine injection. One reason for its inapplicability in this case is the fact that the low concentration of epinephrine (1:25,000) would require the use of a 750-ml. sample in order that a suitable quantity of derivative be obtained.

SUMMARY

In dilute aqueous solutions, such as those used for injection, epinephrine, and norepinephrine are quantitatively converted into chloroform-soluble triacetyl derivatives by the action of acetic anhydride in the presence of sodium bicarbonate. The derivatives may be weighed, identified by the methods of qualitative organic analysis, and a determination made of their specific rotations. From these data may be calculated the quantity of physiologically potent *l*-isomer present in a solution.

Mixtures of the triacetyl derivatives of epinephrine and norepinephrine are quantitatively separable by partition chromatography. This fact may be employed to advantage in the determination of norepinephrine in solutions of U. S. P. epinephrine.

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WHO MAKES IT?

The Registry of Rare Chemicals, Armour Research Foundation of the Illinois Institute of Technology, 35 West 33rd Street, Chicago 16, Ill., seeks information on sources of supply of the following chemicals:

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N-Methylpicolinic acid betaine
Tetrahydro-2-methyl-6-(3-pyridyl)-2H-1,2-oxazine
2,4,6-Trimethylphenol (mesitol)
1,2-Diaminocyclohexane
Phenanthro (1,10,9,8,fghij)perylene (meso-naphthodianthrene)
4-Hydroxy-3-methyl-2-butanone
1-Butanesulfonyl chloride
1-Pentanesulfonyl chloride
2-Methyl-2-butenesulfonyl chloride
3-Methyl-4-oxazolidinecarboxylic acid
3-Methyl-5-oxo-4-oxazolidinecarboxylic acid
3-Methyl oxazolidine
para-Hydroxyphenylacetaldehyde
Solanidine
1-Naphthyl isocyanate
Magnesium lauryl silicate
Cevadine

Prediction of Stability in Pharmaceutical Preparations I. Color Stability in a Liquid Multisulfa Preparation*

By EDWARD R. GARRETT and ROBERT F. CARPER

A procedure for prediction of thermal stability of a component in a complex pharmaceutical preparation is outlined. It is based on the reproducibility of the degradation rate function at the various elevated temperatures and extrapolation of the Arrhenius relation to obtain the rate constants that would describe degradation rates at marketing temperatures. The method is applied to the prediction of color stability at shelf temperatures in a liquid multisulfa preparation and the color intensity is predicted at the end of several years by a study completed within a month's time.

ONE OF THE MOST IMPORTANT legal and moral obligations of the ethical pharmaceutical house is to market pharmaceutical preparations that will maintain their label values and initial appearance for the duration of their market or shelf life. A classical method is to study the stability of shelf-stored pharmaceuticals for the period of time (usually in excess of a year) that the products will be shelved on the normal market. An obvious disadvantage of such a procedure is the loss of time before marketing and the loss of market priority on a competitive product. If the preparation studied by this method is unstable, there is no assurance without a similar time consuming study that a modified product will not be unstable also. Even if the product is released as stable on the basis of such a study, it may be doubtful that the composition is at the economic optimum.

Accelerated studies of stability at higher temperatures have been used as guides in the estimation of shelf life at expected shelf temperatures. Too frequently, such correlations have been intuitive, based on insufficient numbers of elevated temperatures or on empirical relations found in supposedly similar preparations.

Predictions of shelf life from accelerated studies can be placed on a quantitative basis by the application of fundamental physicochemical principles (1). Thermal degradation should follow the Arrhenius relation with absolute temperature (T):

$$k = Ae^{-\Delta Ha/RT} \qquad (1)$$

where k is the specific rate of degradation of the pertinent component, R is the gas constant (1.987 calories degree^{-1} mole^{-1}), A is a constant associated with entropy of the reaction and/or collision factors and ΔH_a is defined as the heat of activation. The logarithmic form of Eq. 1:

$$\log k = -(\Delta H_a/2.303R)\,(1/T) + \text{constant} \qquad (2)$$

shows that any value proportional to the specific rate (k) would permit evaluation of the slope of the plot, $\log k$ vs. $1/T$, and thus the heat of activation. If such a relation among several values determined at elevated temperatures and the absolute temperature is reasonably linear, the prediction of stability at shelf temperatures should be practical.

Degradations in pharmaceutical preparations are frequently complex because of the multitude of components and their possible interactions. It may be economically impractical to study the fundamental mechanisms of degradation, but knowledge of mechanism is not vital for the purposes of stability prediction. A modus operandi would be to determine the degradation or some property of the degradation as a function of time and to linearize the function. For example, rates of change of some property of the degradation at a concentration level may be functions of some power of the concentration of the pertinent component and such rates may be deduced as zero, first, second, etc. order by normal, logarithmic, or reciprocal plots of concentration functions against time. The slopes of such linear plots may be used as estimates of specific rates (k) within the specified concentration ranges. The reproducibility of such a degradation rate function at the various temperatures studied would validate the use of such functions in the prediction of degradation rates at other temperatures.

A frequent pharmaceutical practice is to color preparations for purpose of eye appeal, masking, identification, and association with taste. Such coloring should be maintained throughout the life of the product for fulfillment of the above purposes and also in consideration that the consumer may associate color maintenance with potency maintenance of the product. A dye (carmine: λ max. = 531 mμ, a = 14.25; λ max. = 568 mμ, a = 11.28 at 0.0297 Gm./L. in 50 per cent ethanol, 0.045 N in NaOH) tested in a

* Received January 24, 1955, from the Research Division, The Upjohn Company, Kalamazoo, Mich.
Presented to the Scientific Section, A. Ph. A., Miami Beach meeting, 1955.

liquid multisulfa preparation had shown marked instability and color loss with an undesirable rust-brown discoloration of the product. A new and improved multisulfa product, Citrasulfas® (improved),[1] was to be placed on the market and it was vital that the shelf color stability of this preparation be known as quickly as possible.

The purpose of this study was to determine the color stability of Citrasulfas® by kinetic investigation of the thermally accelerated degradation of the coloring material. The experiment was so designed that it was a measure of the change in color of the product as it would be on the market. There has been no attempt to search for the mechanism for the reaction which would have involved a more detailed research program. The primary aim was to give a prognosis and not a diagnosis in as short a time period as possible and with as little effort as possible. These aims were realized in the brief period of three and one-half weeks.

EXPERIMENTAL

Each 100 cc. of the liquid multisulfa preparation (Citrasulfas®) contained 3.33 Gm. sulfadiazine, 3.33 Gm. sulfamerazine, 3.33 Gm. sulfamethazine, 5.09 Gm. sodium citrate, 0.0397 Gm. FD&C Yellow No. 6, 0.0079 Gm. D&C Red No. 33 in addition to flavoring and suspending agents.

Ten-cc. ampuls were filled with Citrasulfas® by a Baltimore automatic pipetting apparatus and then sealed.[2] Certain of these ampuls were tared and filled weights checked at 2.84 ± 0.03 Gm. to determine accuracy of delivery. The ampuls were placed in 40, 50, 60, and 70° ± 0.3° constant temperature baths, and also at room temperature. The ampuls were withdrawn at intervals, were crushed in a metal beaker and incrementally washed from the broken glass with a total of 80 cc. of pipetted water into a 100-cc. centrifuge tube. The tubes were centrifuged for twenty minutes at 2,000 r. p. m. The supernatant liquid was decanted into a beaker containing 80 cc. 95% ethanol and the solution thoroughly mixed. Spectrophotometric absorbances of the clear solutions were determined in the Beckman spectrophotometer, Model DU, at wavelengths of 350, 375, and from 400 to 600 mμ at 20 mμ intervals against a 50:50 95% ethanol-water blank.

Two fresh ampuls were treated in the above manner. The spectrophotometric readings on the pair showed less than 2% deviation between them at all wavelengths. After standing for two hrs., the alcohol extracted dyes showed no change in absorbance. Half of the concentration gave half the absorbances, demonstrating the gross validity of Beer's law.

FDC Yellow No. 6 at 0.0342 Gm./L. of 50% ethanol had a visible spectrophotometric maximum at 487 mμ, the absorptivity (a) was 44.4; a flex at 504 mμ, a = 40.7; and a flex at 425 mμ, a = 21.9. The absorptivities at 480, 500, and 520 mμ were 43.0, 41.4, and 31.9 respectively.

D&C Red No. 33 at 0.03838 Gm./L. of 50% ethanol had a visible spectrophotometric maximum at 537 mμ and the absorptivity (a) was 53.7. Several minor maxima were at 435, 400, and 378 mμ and the respective absorptivities were 10.42, 11.20, and 11.05. The absorptivities at 480, 500, and 520 mμ were 22.8, 36.9, and 49.9 respectively.

RESULTS AND DISCUSSION

Figure 1 at 60° is an example of the decrease in spectral absorbance of Citrasulfas®. The nature of the change in the curves with time was similar at all the studied temperatures. The rate of change of the observed absorbance of the extracted color is a valid measure of the thermal degradation although the absolute values of the absorbance ordinate may have no inherent significance. The absorption band (480–520 mμ) is what gives the preparation its decided pink color. Decrease in the magnitude of this band decreases the visual sharpness of the color. It is apparent that thermal effects decrease the color by decreasing the band's magnitude in this visible region but increase the absorption in the near and ultraviolet regions.

Plots of observed absorbances at 500 mμ vs. time in hours for the various temperatures studied are given in Fig. 2. Their adherence to linearity shows that the slopes of these lines are excellent criteria of the rates of decrease in absorbance with time. The curves and slopes are of the same magnitude at 480 mμ. Table I is a summary of these rates.

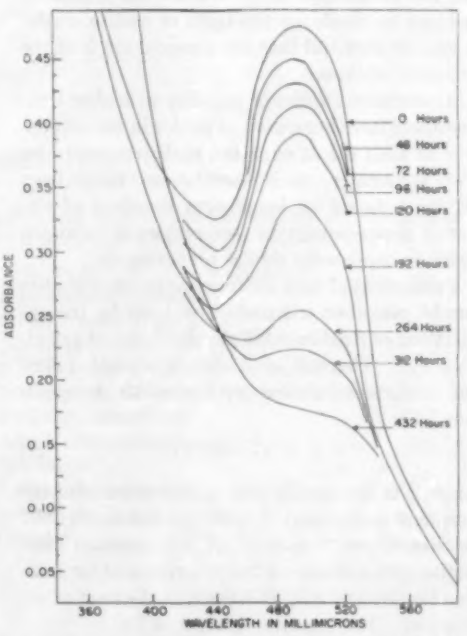

Fig. 1.—The effect of temperature (60°C.) on the visible absorption spectra of a colored liquid multisulfa preparation; plot of optical absorbance of extracted color against wavelength in mμ at various times.

[1] Trade name of The Upjohn Company.

[2] The cooperation of Victor F. Dargitz is gratefully acknowledged.

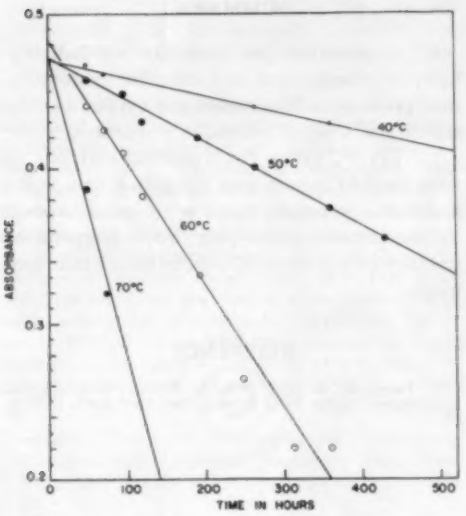

Fig. 2.—Plot of optical absorbance of extracted color against time at 40, 50, 60 and 70°C. at 500 mμ. Room temperature samples had absorbances of 0.470 ± 0.005 throughout the duration of the study.

TABLE I.—RATES OF DECREASE OF ABSORBANCE OF PREPARED EXTRACT AT 500 mμ PER HOUR (k)

°C.	(k)
40	0.00011
50	0.00028
60	0.00082
70	0.00196

To predict the absorbance (A_t) of the extract at t hours, the k appropriate to the temperature should be used and the absorbance of the color extract at zero time ($A_0 = 0.470$) in the equation:

$$A_t = A_0 - kt \qquad (3)$$

At 460 mμ and 520 mμ, the best linearity is obtained with a first order plot (i. e., log absorbance vs. time in hours). These first order rate constants (k_1) could be evaluated from such plots according to the equation

$$\log A_t = -k_1 t/2.303 + \log A_0 \qquad (4)$$

where $-k_1/2.303$ is the slope of the plot. The k_1's evaluated in such a manner are given in Table II.

TABLE II.—FIRST ORDER RATE CONSTANTS AT 460 AND 520 mμ

Temp. °C.	k_1 at 460 mμ	k_1 at 520 mμ
50	0.53×10^{-3} hr^{-1}	0.852×10^{-3} hr^{-1}
60	1.51×10^{-3} hr^{-1}	2.16×10^{-3} hr^{-1}
70	4.4×10^{-3} hr^{-1}	7.1×10^{-3} hr^{-1}

The A_t for a given temperature at the desired wavelength at a given time (t) in hours can be calculated from Eq. 4.

The data in either Table I or Table II could be used in the prediction of absorbance at given times at lower temperatures. The data in Table I at the maximum absorption will be discussed more fully in this paper. However, it is to be noted that predictions based on Table II using the same methods of mathematical analysis (except for using Eq. 4 rather than Eq. 3) gave comparable results.

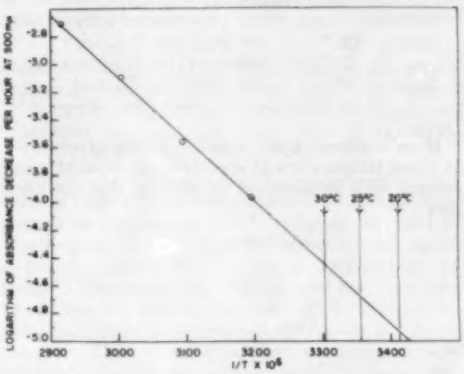

Fig. 3.—Plot of the logarithm of the rate of decrease in absorbance of extracted color per hour against the reciprocal of the absolute temperature (T) as obtained at 500 mμ.

From the data of Table I, a logarithmic plot of the Arrhenius expression, Eq. 1, is given in Fig. 3. The linear nature of the resultant curve is excellent verification of the method's utility in prediction of rates of degradation at lower temperatures.

The equation of the curve of Fig. 3 corresponding to Eq. 2 is:

$$\log k = -4.46 \times 10^3/T + 10.3 \qquad (5)$$

The heat of activation,[3] $\Delta Ha = 20.4$ Kcal./mole, is comparable to that of many thermal reactions[4] and substantiates the equation's validity.

Thus Eq. 5 or comparable points on the curve in Fig. 3 can be used to determine k values for lower temperatures. Some of these are listed in Table III.

TABLE III.—k VALUES DETERMINED FROM ARRHENIUS EXPRESSION

°C.	k, Absorbance dec./hr.
20	0.0000115
25	0.0000209
30	0.0000363

These k values may then be used in Eq. 3 to determine the times when the color has reached unde-

[3] The slopes of the Arrhenius' plots for the first order rate constant data of Table II are also 4.5×10^3, similar to the slope in Eq. 5 for the zero order rate constant data of Table I.

[4] A common statement is that a reaction rate increases by a factor of 2 or 3 for each 10° rise in temperature (1). Thus for 20 and 30° C.:

$$\log k_{30°}/k_{20°} = -4.5 \times 10^3 \times (1/T_{30°} - 1/T_{20°})$$
$$= -4.5 \times 10^3 \times 10^{-4}\,(3.33 - 3.41)$$
$$= 0.36$$
$$\text{Thus, } k_{30°}/k_{20°} = 2.3$$

sirably low intensity. Observation of the ampuls degraded at higher temperatures had shown that an absorbance of the extract of $A_t = 0.225$ should be deemed undesirable. The predicted times of this estimated point of rejection are given for several temperatures in Table IV.

TABLE IV.—DURATION OF COLOR (CORRESPONDING TO ESTIMATED POINT OF REJECTION OF ABSORBANCE OF EXTRACT, $A_t = 0.225$)

20°	887 days (ca. 2½ yrs.)
25°	488 days (ca. 1⅓ yrs.)
30°	281 days (ca. ¾ yr.)

If an arbitrary color stability period of one year at room temperature is specified, the studied preparation may be accepted as meeting this specification.

SUMMARY

1. A procedure for prediction of shelf stability of a component in a complex pharmaceutical preparation is outlined and applied to color stability of a liquid multisulfa preparation.

2. FD & C yellow No. 6 and DC Red. No. 33 were used to impart pink coloration to a liquid multisulfa preparation and were found to meet the specification of the year's room temperature stability by a study that took no longer than four weeks.

REFERENCE

(1) Frost, A. A., and Pearson, R. G., "Kinetics and Mechanisms," John Wiley & Sons, Inc., New York, 1953, p. 23.

Book Notices

This is Liquid Sugar. A Technical Guide for the Liquid Sugar User. Refined Syrups & Sugars, Inc., Yonkers, New York, 1955. VII + 205 pp. 15.5 x 23 cm.

A monograph on "liquid sugar" describing production, testing, properties, and commercial applications of the generally saturated aqueous solutions of sugar (sucrose, invert, or mixtures of both). "Syrup" is often used synonymously with "liquid sugar," but they are distinguished industrially by the U. S. Sugar Act definition of a liquid sugar as a solution containing less than six per cent soluble nonsugar solids. Technical data on specific gravity, density equivalents, turbidity, alteration of concentration, determination of saturation relations of sucrose, invert sugar and water, viscosity and boiling points, are tabulated in the appendix.

New Practical Formulary. By MITCHELL FREEMAN, Chemical Publishing Company, Inc., New York, 1955. 376 pp. 14 x 21.5 cm. Price $7.95.

This is an eminently practical book which contains in just one volume useful information in such varied fields as agricultural specialties; animal preparations; cements, glues, mucilages; cosmetics, perfumes; disinfectants; insect and rodent destroyers; pharmaceutical and proprietary preparations; and stain removers. Earlier books in this same field have been less concise and sometimes ran into several volumes, whereas Mr. Freeman seems to be able to present a cornucopia of useful up-to-date information in one handy volume.

Particularly useful is the appendix which contains the names of various sources of supply for the material needed in the preparation of the various formulas listed. This book deserves a place on the shelves of every drug store.

Pharmazeutisches Wörterbuch. By CURT HUNNIUS. Walter de Gruyter & Co., Berlin, 1955. xi + 610 pp. 14 x 19.5 cm. Price DM 18.50.

This second edition of Hunnius' dictionary has been considerably increased in contents and volume over the first one. The more than one hundred additional pages have been used partly to supply additional material on topics previously discussed and partly to bring the book up to date by including the latest developments in the field of pharmacy. A happy medium has been found between short definitions covering very specific topics and articles running to as many as three pages discussing larger ones in such a way as to serve as an introduction to the uninitiated. Among the new tables included, we find indicators, the numbers of organic ring compounds, doses for infants and children, and the vitamin contents of certain foods. Particular attention may also be called to the table of biological units which was contained in the first issue and as far as we know has no counterpart in English. For everybody who has to deal with pharmaceutical material this book is indispensable. It shows the remarkable comeback which Germany has made in the field of pharmacy.

Management of Addictions. Edited by EDWARD PODOLSKY. The Philosophical Library, New York, 1955. xvii + 413 pp. 15 x 22.3 cm. Price $7.50.

This book is a compilation of papers on various aspects of addicition to alcohol and various drugs. Mechanisms of addiction are discussed from the biophysical and psychological viewpoints, and therapeutic treatments are described. Addiction to alcohol constitutes the greatest single addiction problem, and twenty-six of the thirty-five papers are devoted to that subject. The tendency to consider barbiturate habituation as a true drug addiction is evident in a number of the papers. The book is intended primarily for physicians but it should interest others who are concerned with this problem. The value of the book as a reference is limited by the absence of a subject index.

Consulting Services. Association of Consulting Chemists and Chemical Engineers, Inc., New York, 1955. xxiv + 110 pp. 15 x 22 cm. Price $1.

The fifteenth edition of "Consulting Services" (1955) published by the Association of Consulting Chemists and Chemical Engineers, Inc., now appears in a new format, 6 x 9, for easy handling, and contains 144 pages of valuable information. It is divided into three sections.

Section I. *Classifier.* This section contains over 200 items of activity, with the members specializing or qualifying in the given field indicated by key numbers, which correspond to the scope pages in Section II. In this section each member's qualifications and activities are given.

Section III, the index, lists members alphabetically, including names of organizations with which they are affiliated. Geographical locations of domestic and foreign branch offices, earmarked by an asterisk, are also indicated.

Antibiotics Annual, 1954–1955. Proceedings of the Second Annual Symposium on Antibiotics. HENRY WELCH and FÉLIX MARTI-IBÁÑEZ, Editors. Medical Encyclopedia, Inc., New York, 1955. ix + 1154 pp. 18 x 26 cm. Price $10.

This book is a compilation of the one hundred and seventy papers presented at the second annual Symposium on Antibiotics held October 25–29, 1954 in Washington, D. C. The Symposium was again sponsored jointly by the U. S. Department of Health, Education, and Welfare, and the Food and Drug Administration, Division of Antibiotics, in collaboration with the journal *Antibiotics & Chemotherapy.* The compilation makes much recent work in this field conveniently available as a reference source. Adequate subject and authors indexes are appended to facilitate the search for major and minor subjects covered in the papers.

BOOKS RECEIVED

The Biologic Effects of Tobacco. ERNEST L. WYNDER, Editor. Little, Brown and Company, Boston, 1955. xiii + 215 pp. 14.5 x 21 cm. Price $4.50.

The Roger Adams Symposium. Papers presented at a symposium in honor of Roger Adams at the University of Illinois, September 3 and 4, 1954. John Wiley & Sons, Inc., New York, 1955. ix + 140 pp. 15.5 x 23.5 cm. Price $3.75.

Microbiology. 2nd ed. By FLORENE C. KELLY and K. EILEEN HITE. Appleton-Century-Crofts, Inc., New York, 1955. xi + 615 pp. 17 x 23.5 cm. Price $7.50.

Origins of Resistance to Toxic Agents. Proceedings of the symposium held in Washington, D. C., March 25–27, 1954. M. G. SEVAG, ROGER D. REID, and ORR E. REYNOLDS, Editors. Academic Press, Inc., New York, 1955. xv + 471 pp. 16 x 23.5 cm. Price $12.

Analysis of Insecticides and Acaricides. A treatise on sampling, isolation, and determination, including residue methods. By FRANCIS A. GUNTHER and ROGER C. BLINN. Interscience Publishers, Inc., New York, 1955. xi + 696 pp. 16 x 23.5 cm. Price $14.

General State Food and Drug Laws. Food Law Institute Series. Annotated. By DAVID H. VERNON and FRANKLIN M. DEPEW. Commerce Clearing House, Inc., Chicago, 1955. xii + 804 pp. 17.5 x 25 cm. Price $17.50.

Special Federal Food and Drug Laws. Food Law Institute Series. Annotated. By THOMAS W. CHRISTOPHER and CHARLES WESLEY DUNN. Commerce Clearing House, Inc., Chicago, 1955. xiii + 1334 pp. 17.5 x 27.5 cm. Price $26.50.

Advances in Enzymology and Related Subjects of Biochemistry. Vol. 16. Edited by F. F. NORD. Interscience Publishers, Inc., New York, 1955. v + 584 pp. 16.5 x 23.5 cm. Price $11.

Corrections

WHO Recommended International Non-Proprietary Names

The introductory paragraph to *World Health Organization Recommended International Non-Proprietary Names* [THIS JOURNAL, 44, 387(1955)], should read:

In accordance with paragraph 7 of the Procedure for the Selection of Recommended International Non-Proprietary Names for Pharmaceutical Preparations, notice is hereby given that the following are selected as recommended international non-proprietary names.

The inclusion of a name in this list does not imply any recommendation for the use of the substance in medicine or pharmacy.

Essential Elimination of Sodium Cycloheptenylethylbarbiturate (Medomin®) in Rabbits

To the Editor:

In the article entitled "Essential Elimination of Sodium Cycloheptenylethylbarbiturate (Medomin®) in Rabbits," by Theodore Koppanyi, Charles Morgan, and Joseph Princiotto, [THIS JOURNAL, 44, 221(1955)], the following corrections should be made.

In the second line of the right hand column, page 223, change the words *cycloheptenylethylbarbituric acid* to read *cycloheptenonylethyl barbituric acid*, and at the end of the sentence add "This pharmacologically inert compound may also be recovered following the administration of Nembutal® [Maynert, E. W., and van Dyke, H. B., *Science*, **110**, 661(1949)]."

Under "References," page 223, change the page number in reference (4) from "10" to "101."

THEODORE KOPPANYI

Compressed Tablets: Weight Variation

To the Editor:

In the article entitled "Compressed Tablets: Weight Variation" by Angel S. Armabulo, Helena Suen Fu, and Dwight L. Deardorff [THIS JOURNAL 42, 692(1953)], a sentence on page 693 reads as follows: "The constant D_4 has the value 2.11 for subgroups of 4." The value 2.11 applies to subgroups of 5. The sentence should read: "The constant D_4 has the value 2.28 for subgroups of 4." The correct value was used in the computations in the article.

DWIGHT L. DEARDORFF

A Taste Panel Study of the Saccharin Off-Taste

To the Editor:

In our first paper on "A Taste Panel Study of the Saccharin Off-Taste,"[1] certain sweetness values for saccharin sodium solutions should be corrected. The values in error were given in the Abstract and in the Conclusions. A 0.026% saccharin sodium solution approximately equals the sweetness of a 3.6% sucrose solution instead of 10%. An extrapolation of results from all completed tests indicates that 0.07% solution of saccharin sodium would approximately equal the sweetness of a 4.6% sucrose solution instead of 28%. The extrapolation is based on a straight line relationship which seems to exist between sweetness, expressed in per cent of sucrose, and the logarithm of the concentration of saccharin sodium. This logarithmic relationship is now being studied further, and will be discussed in a later communication.

The higher sweetness values given originally were calculated from sweetness factors determined at or near the sweetness threshold. We knew that the sweetness factor decreases as the concentration of saccharin is increased, but we did not then realize that the drop has as steep a slope as was later demonstrated.

We believe that this correction should be made as an aid to those who might want to make a practical application of the data we have presented.

FRED J. HELGREN
MATTHEW J. LYNCH
F. J. KIRCHMEYER

[1] THIS JOURNAL, 44, 353(1955).

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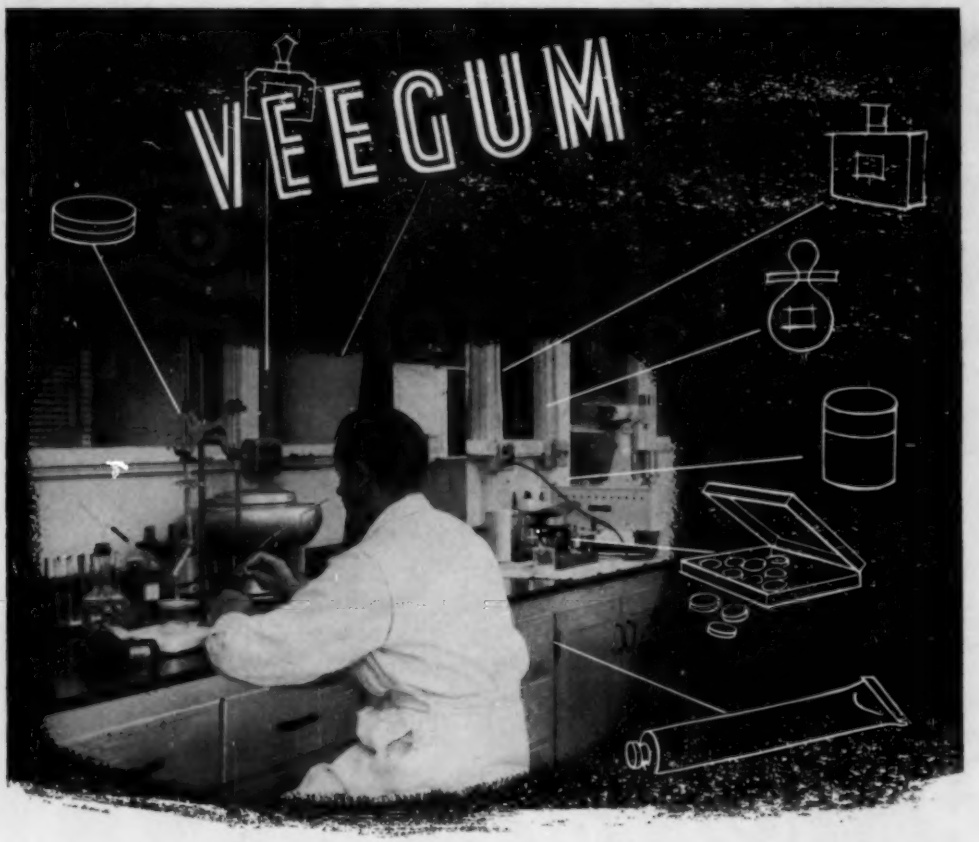

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